PhD Thesis

Immune cell interactions with stellate cells modulating HBV-related liver fibrosis

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Declaration of Authorship

I, Harsimran D Singh, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Chronic infection with hepatitis B virus is a global health issue, leading to liver cirrhosis and hepatocellular carcinoma that accounts for more than six hundred thousand deaths annually. Recent work has focused on the role of Natural killer (NK) cells in liver fibrosis in other contexts. In CHB, NK cells have been shown to play an anti-viral as well as an immunoregulatory role. Here we investigate the role of NK cells in the context of CHB-driven liver fibrosis, using primary hepatic stellate cells (HSC) isolated from healthy human liver margins. Our results demonstrate that NK cells from CHB patients have a highly variable and limited potential to kill hepatic stellate cells, which is increased in patients with progressive liver fibrosis, is further enhanced by interferon-alpha pre-activation *in vitro* and is abrogated by antiviral therapy with nucleos(t)ide inhibitors. We have explored the role of the death ligand TRAIL expressed on NK cells in inducing apoptosis of stellate cells that express high levels of the death-inducing receptor TRAIL-R2. We observed that blockade of TRAIL on the NK cells was only able to reduce killing of HSC in selected cases but not in the whole cohort, suggesting other levels of regulation. Moreover TRAIL ligand (*SuperKiller*TRAIL) treatment of primary HSC did not induce a degree of apoptosis commensurate with their high expression of the death-inducing receptor TRAIL-R2. We therefore probed the expression of the inhibitory receptors TRAIL-R3 and R4. We found expression of these receptors on primary human HSC both *ex vivo* and after *in vitro* culture. The level of expression of TRAIL-R4 showed a remarkably strong correlation with the susceptibility of primary HSC to undergo apoptosis.

Furthermore, blockade of these inhibitory receptors rendered primary HSC more susceptible to apoptosis by *SuperKiller*TRAIL and by TRAIL-expressing NK cells. Our results are the first to demonstrate a functional role for TRAIL-R3 and -R4 in regulating susceptibility to apoptosis in primary cells. These novel findings will help us to define potential biomarkers and therapeutic targets for liver fibrosis.

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αSMA	alpha smooth muscle actin
ADCC	Antibody-dependent cellular cytotoxicity
Adv	Adefovir
ALT	Alanine transaminase
APC	Antigen presenting cells
Bid	Bcl2- interacting domain death agonist
Bim	Bcl2- interacting mediator
BLQ	Below levels of quantification
Breg	Regulatory B cell
C4	Complement 4
C5	Complement factor 5
CCR7	Chemokine (C-C motif) receptor 7
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
c-FLIP	Cellular FLICE inhibitory protein
CHB	Chronic hepatitis B virus infection
CTGF	Connective tissue growth factor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCR6	CXC-chemokine receptor 6
DC	Dendritic cells
DD	Death domain
DED	Death effector domain
DNA	Deoxyribonucleic acid
EASL	European Association for the Study of the Liver
EMT	Epithelial mesenchymal transition
ER	Endoplasm reticulum
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FADD	Fas associated protein with death domain

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GFP	Green fluorescent protein
HA	Haemagglutinin
HBV	Hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B precore-core antigen
HBx	Hepatitis B X protein
HBsAg	Hepatitis B surface antigen
HCC	Hepatocellular cancer
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSC	Hepatic stellate cell
IFNα	Interferon α
IFNγ	Interferon γ
ILC	Innate lymphoid cell
iNK	Immature natural killer
iNKT	invariart natural kiler T
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IL	Interleukin
JAK	Janus kinase
KIR	Killer immunoglobulin receptor
KLRG-1	Killer cell lectin-like receptor subfamily G, member 1
KC	Kupffer cells
Lam	Lamivudine
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
MAIT	Mucosal-associated invariant T
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDSC	Myeloid-derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKT	Natural killer T
NKG2A	Natural-killer group 2, member A
NKG2C	Natural-killer group 2, member C
NKG2D	Natural-killer group 2, member D
NKp30	Natural killer cell p30-related protein
NKp44	Natural killer cell p44-related protein
NKp46	Natural killer cell p46-related protein
NPC	Non-parenchymal cells
NTCP	Sodium taurocholate cotransporting polypeptide
OPG	Osteoprotegerin
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PCR	Polymerase chain reaction
PD-1	Programmed death – 1
PDGF	Platelet derived growth factor
PGK	Phosphoglycerate kinase
pgRNA	Pre-genomic RNA
Peg-IFNα	Pegylated Interferon α
PI3K	Phosphoinositide 3-kinase
PLC-γ	Phospholipase C gamma
PRR	Pattern recognition receptor
rcDNA	Relaxed circular DNA
RIP1	Receptor interacting protein 1
RNA	Ribonucleic acid
RORγT	Retinoic acid receptor related orphan receptor gamma
ROS	Reactive oxygen species
RT PCR	Real time polymerase chain reaction
sh	short hairpin
SHP-1	Src homology 2 domain-containing phosphatase-1

SLT	Secondary lymphoid tissue
SNP	Single nucleotide polymorphism
SVP	Sub-viral particles
Ten	Tenofovir
TGFβ	Transforming growth factor beta
Tim-3	T cell immunoglobulin mucin-3
TIMP	Tissue inhibitor of mettaloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TRADD	TNF-receptor associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TRAIL R	TNF-related apoptosis inducing ligand receptor
Treg	Natural regulatory T cells
WHO	World health organization
WHV	Woodchuck hepatitis virus
ZAP-70	Zeta-chain-associated protein kinase 70
7AAD	7-aminoactinomycin D

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List of Publications

Publications

1. Macdonald DC, **Singh H**, Whelan MA, Escors D, Arce F, Bottoms SE, Barclay WS, Maini M, Collins MK, Rosenberg WM. Harnessing alveolar macrophages for sustained mucosal T-cell recall confers long-term protection to mice against lethal influenza challenge without clinical disease. Mucosal Immunol. 2014 Jan;7(1):89-100. doi: 10.1038/mi.2013.27. Epub 2013 May 29.

2. Schurich A, Pallett LJ, Lubowiecki M, **Singh HD**, Gill US, Kennedy PT, Nastouli E, Tanwar S, Rosenberg W, Maini MK. The third signal cytokine IL-12 rescues the anti-viral function of exhausted HBV-specific CD8 T cells. PLoS Pathog. 2013 Mar;9(3):e1003208. doi: 10.1371/journal.ppat.1003208. Epub 2013 Mar 14.

3. Peppa D, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, Micco L, Nebbia G, **Singh HD**, Adams DH, Kennedy PT, Maini MK.Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion.J Exp Med. 2013 Jan 14;210(1):99-114. doi: 10.1084/jem.20121172. Epub 2012 Dec 17.

4. Nebbia G, Peppa D, Schurich A, Khanna P, **Singh HD**, Cheng Y, Rosenberg W, Dusheiko G, Gilson R, ChinAleong J, Kennedy P, Maini MK. Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. PLoS One. 2012;7(10):e47648. doi: 10.1371/journal.pone.0047648. Epub 2012 Oct 24.

1. Introduction

The liver is one of the most resilient organs of the human body. It can endure chronic injury and undergo repair over extended periods of time. When infected with a non-cytopathic virus such as Hepatitis B virus (HBV), the immune response elicited in response to infection causes liver injury. In chronic infections this can lead to years of continued liver damage, which in turn leads to cirrhosis and/or hepatocellular carcinoma (HCC). The liver myofibroblasts, in particular hepatic stellate cells (HSC), produce extra-cellular matrix that forms the architecture of the liver while new cells are being generated. In chronic injury these cells deposit new collagen that leads to pathological liver fibrosis. The cellular elements and biological mechanisms involved in liver fibrosis have been described and novel treatments directed at preventing or slowing fibrosis are now entering clinical trials (Ikenaga, 2013).

In chronic viral hepatitis evidence from randomized controlled clinical trials has demonstrated that eradication of hepatitis C virus (HCV) (Poynard et al., 1998) or control of HBV infection (Marcellin et al., 2013) can result in reversal of liver fibrosis. However in HBV infection, control of vireamia with nucleotide or nucleoside analogues does not result in viral clearance and so the manipulation of immunity to clear HBV without causing liver damage has been a goal of research in recent decades. The role of immune response in liver fibrosis has increasingly become the focus of research in recent years and understanding these interactions will help us understand the progression of fibrosis in chronic HBV infection better.

Liver microanatomy

The liver is an organ of hemodynamic confluence, which receives blood from the hepatic artery and portal vein. Blood from the portal vein carries by-products of digestion including nutrients and food antigens from the intestines. The arterial and venous blood mingle in the liver leading to hypoxic conditions and slow blood flow in the microvessels also known as the liver sinusoid. Micro-anatomically, the liver comprises of a meshwork of sinusoids where an exchange of nutrients occurs between the hepatocytes and blood (Thomson and Knolle, 2010). As illustrated in Figure 1.1, the hepatocytes, which are the main parenchymal cells of the liver, are separated from the hepatic sinusoid by non-parenchymal cells (NPC). The liver sinusoidal endothelial cells (LSECs) line the sinusoid with fenestrations around them. In between the LSECs and the hepatocytes lie the HSC. The kupffer cells (KCs), which are the liver resident macrophages, along with liver resident DC lie in the sinusoid and are in direct contact with the blood and therefore serve an important antigen presentation function in the liver (Thomson and Knolle, 2010). The intra-hepatic lymphocytes (IHL) also lie in the sinusoid and are able to migrate through the fenestrations in a healthy liver (Crispe, 2009).

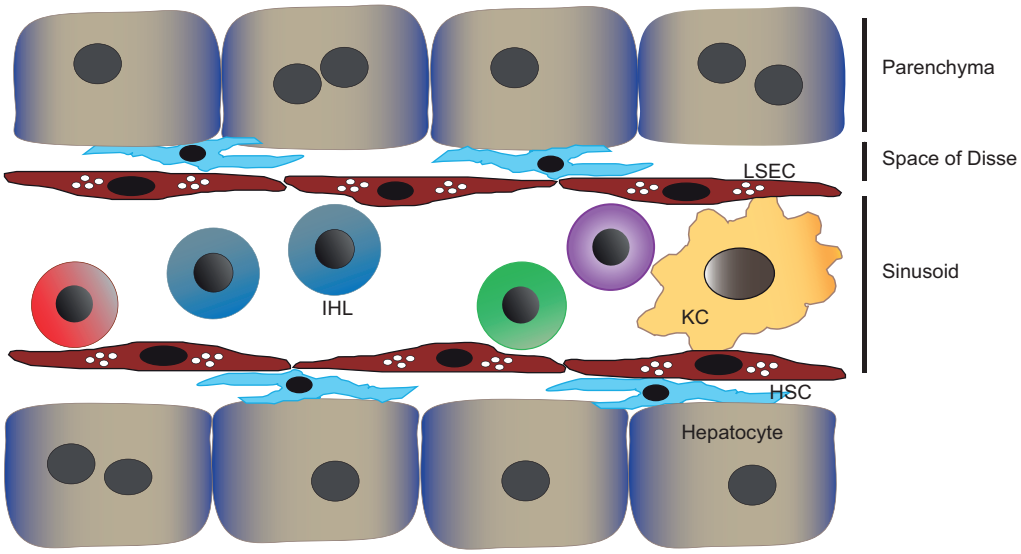


Figure 1-1: Liver microanatomy.

This diagram illustrates the microanatomy of the liver sinusoidal space. Hepatocytes form the parenchyma of the liver and are supplied with blood by a fine meshwork of narrow-lumen vessels making up the sinusoidal vasculature. The space between the sinusoid and parenchyma, within which HSC lie, is referred to as the Space of Disse. The sinusoid is lined with LSECs. Liver resident macrophages, KC and IHL lie in the sinusoid where they come in direct contact with the blood.

Hepatitis B infection – influence on public health and treatment

One of the most common causes of liver disease world-wide is infection with HBV. Chronic infection with HBV (CHB) leads to liver inflammation and fibrosis, which over an extended period of time leads to severe liver disease such as cirrhosis and hepatocellular carcinoma (HCC). According to the World Health Organisation (WHO) an estimated six hundred thousand people die due to these complications annually making chronic hepatitis B (CHB) a problem of great importance for global public health. In 2012, mortality associated with HBV infection ranked 15th in the global causes of death (Lozano et al., 2012). According to WHO reports, an estimated two billion people are infected with HBV, of which around 350 million have persistent infection. HBV can be transmitted vertically (perinatal route) or horizontally (parenteral or sexual routes). Most adults infected with HBV are able to resolve infection as the result of vigorous and effective antiviral immune responses, leading to lifetime immunity (Penna et al., 1996; Rehermann et al., 1996; Rehermann et al., 1995). However, HBV transmitted from mother to child neonatally or acquired in infancy commonly results in chronic infection which in turn leads to CHB (Liaw and Chu, 2009). Even though a prophylactic vaccine for HBV does exist and is very effective, it is futile as a therapeutic vaccine (Lavanchy, 2004).

The global incidence of HBV infection can be stratified geographically based on areas of high, moderate and low prevalence. West Africa, South-East Asia, Western Pacific and Eastern Europe make up areas of high prevalence (5-13%) (Liaw and Chu, 2009; Ott et al., 2012). Rest of Europe, South America,

Australia and Japan are areas of moderate frequency (2-4%) and Central America that of low frequency (<2%) (Ott et al., 2012). Majority of cases in areas of low prevalence of infection are by the horizontal route whereas most individuals infected in the high prevalence areas are infected vertically (Lavanchy, 2004).

For CHB, targets for therapy include suppression of viral replication, immunomodulation leading to remission of biochemical changes in the liver and prevention of liver damage which eventually cause cirrhosis and HCC. Currently there are two types of drugs being used to treat CHB, the first are nucleot(s)ide analogs that are anti-viral and are administered orally and the second is the pegylated form of Interferon-α (Peg-IFN-α) which has both antiviral and immunomodulatory effects and is administered subcutaneously (Papatheodoridis et al., 2008b). Oral anti-viral drugs, Entecavir and Tenofovir have improved the treatment of CHB as they have a high barrier to genetic resistance; however, clearance of the virus is rarely achieved (Papatheodoridis et al., 2008b). Even though treatment with peg-IFN-α is short-term (12 months), only a minority of patients achieve lasting virologic control or immune seroconversion (Papatheodoridis et al., 2008b). Patients with genotypes A and B respond better than those with genotype C and D when treated with peg-IFN-α but no genotypic difference are observed in patients treated with oral anti-virals (Lok and McMahon, 2007).

HBV replication, natural history and clinical course

HBV belongs to the *Hepadnaviridae* family of viruses with a small DNA genome of ~3200 nucleotides. It is a hepatotropic virus that infects and replicates in the hepatocytes that make up most of the parenchyma of the liver. HBV has a spherical structure. The HBV genome is made of relaxed circular partially double-stranded DNA that encodes four overlapping open reading frames (ORFs): the pre S/S encoding the three viral surface proteins, the precore/core encoding the core, forming the antigenic structure of the viral capsid and the non-structural precore, as secreted hepatitis B e-antigen (HBeAg); the pol ORF encoding the polymerase, which includes transcriptases that are necessary for RNA encapsidation and DNA synthesis; and the X protein (HBx), essential for viral replication (Dandri and Locarnini, 2012) (Figure 1.2). Towards the exterior of the genome are the three envelope proteins of different sizes; large (L), medium (M) and small (S) that share the same C-terminal domain and contain the hepatitis B surface antigen (HBsAg) (Glebe and Urban, 2007).

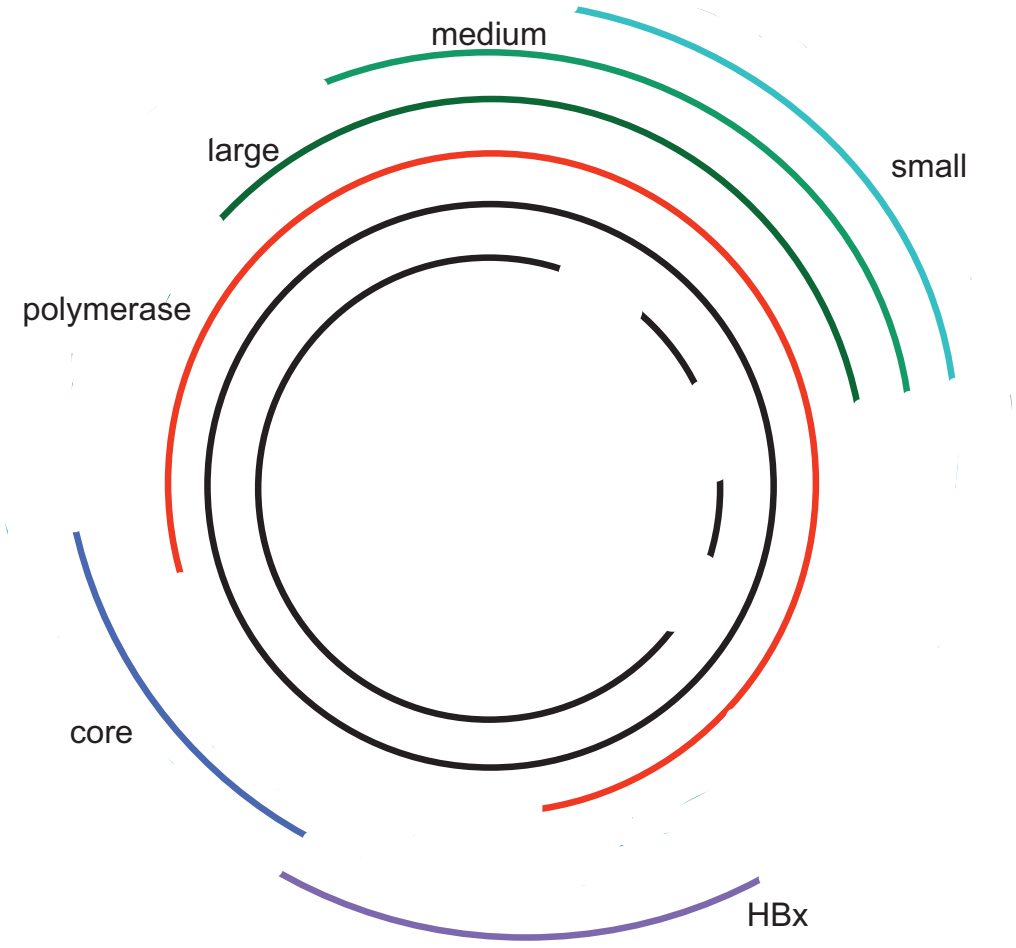


Figure 1-2: HBV genome.

The genome of HBV is 3.2kb. It encodes four overlapping ORFs. In blue is precore/core encoding HBeAg and HBcAg. In red is the viral Pol, green illustrates the S,M and L env ORFs and finally the HBx ORF is shown in purple.

HBV infects hepatocytes. The point of entry of virions into the cells has been recently described to be sodium taurocholate cotransporting polypeptide (NTCP) (Yan et al., 2012). Once in the cell cytoplasm, the virions are uncoated and the relaxed circular DNA (rcDNA) is released into the nucleus via the nuclear pore complex (Schmitz et al., 2010). Inside the nucleoplasm, the viral rcDNA depends on the host cells replicative machinery to remove and repair terminal proteins and complete the synthesis of the positive DNA strand to yield a covalently closed circular (ccc) supercoiled DNA molecule (Urban et al., 2010). cccDNA undergoes transcription essential to produce proteins and facilitate viral replication in the cytoplasm after reverse-transcription of the pre-genomic RNA (pgRNA) (Nassal, 2008). In the cytoplasm, the viral RNAs serve as mRNAs to translate into viral structural and non-structural proteins: HBV surface, core, polymerase and X proteins (Block et al., 2007; Summers, 1988). Inside these capsids, the RNA undergoes reverse transcription to produce the first single stranded viral cDNA which acts as a template for the synthesis of the second strand and leads to the formation of the relaxed circular double stranded DNA genome. These mature DNA containing capsids are transported bi-directionally within the cytoplasm: one pathway ends at the endoplasmic reticulum (ER) where they interact with the envelope proteins to initiate budding which results in the formation and secretion of non-infectious sub-viral particles (SVP) and infectious virions that are released from the cell; the other pathway transports them back into the nucleus to expand the cccDNA pool (Dandri and Locarnini, 2012; Urban et al., 2010). The replication cycle of HBV is illustrated in figure 1.3.

Lack of proofreading of viral polymerases and mutations in cccDNA give rise to genetic heterogeneity of HBV (Balmasova et al., 2014; Locarnini and Zoulim, 2010). These mutations allow the virus to evade and escape host immune-mediated viral clearance and anti-viral drugs. There are ten known HBV genotypes with described geographical prevalence (Balmasova et al., 2014). HBV genotypes influence the clinical outcome of the disease and response to anti-viral drugs (Tanwar and Dusheiko, 2012).

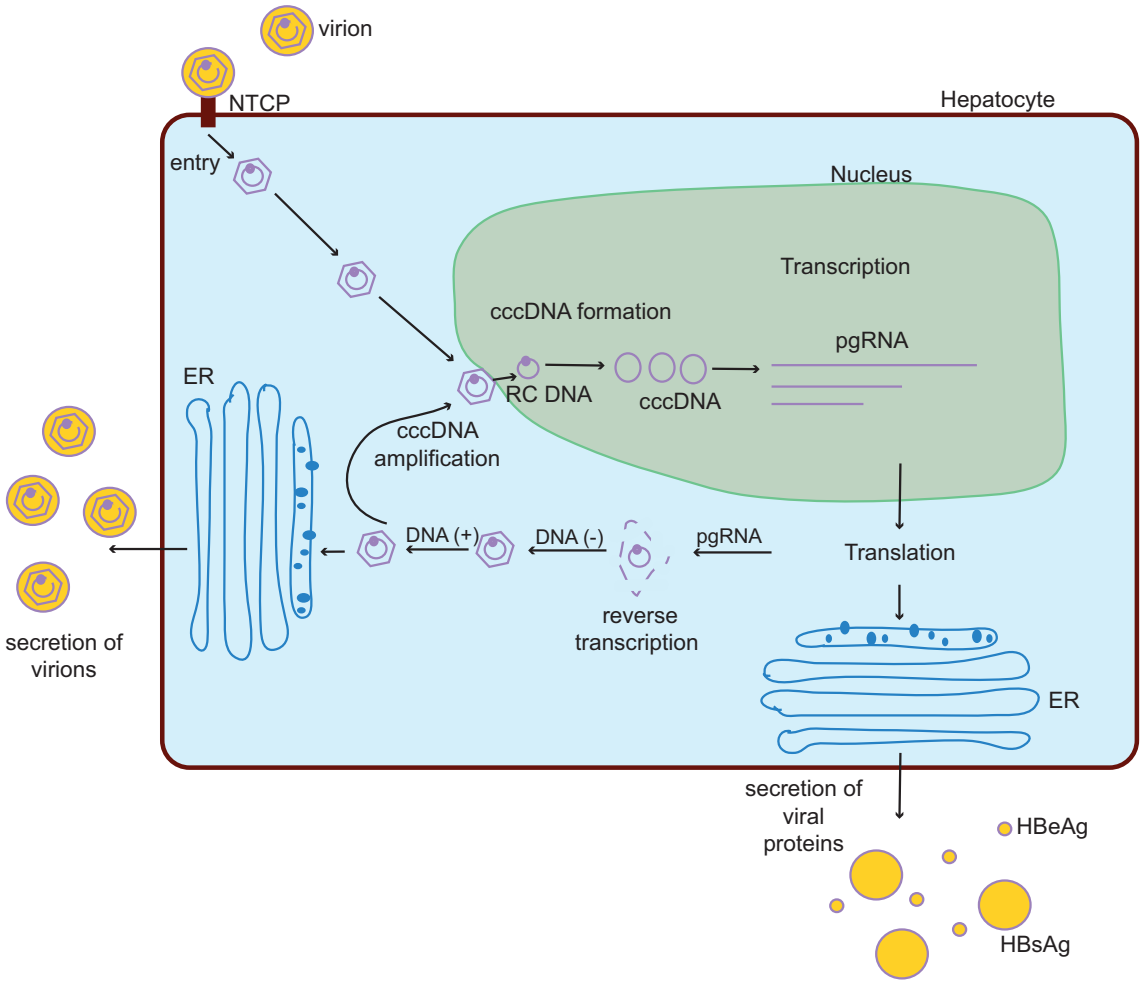


Figure 1-3: HBV life cycle.

The HBV virion enters hepatocytes through NTCP. In the cytoplasm the virion uncoats and the rcDNA is released into the nucleus. In the nucleus, the rcDNA is synthesized to form ccc DNA. cccDNA undergoes transcription to produce proteins to facilitate viral replication in the cytoplasm. In the cytoplasm, the viral RNA is translated into viral structural and non-structural proteins, eventually leading to the formation of viral cDNA which acts as a template for formation of rcDNA. The mature DNA containing capsid is transported bi-directionally in the cytoplasm; one towards ER to form and secrete SVP and the other back to the nucleus to expand the pool of cccDNA.

Along with the SVP, HBV replication in an infected cell also leads to the secretion of non-structural part of the nucleoprotein, HBeAg. This antigen has no role in viral replication, however it has been suggested to have immune-modulating functions (Wieland and Chisari, 2005). HBeAg and HBsAg have been shown to skew the adaptive immune response in HBV mouse models and may contribute to T cell exhaustion (Chen et al., 2005; Guidotti and Chisari, 2006). Studies done by some groups have demonstrated that these secreted viral particles have the capacity to suppress toll-like receptor (TLR) mediated innate immune responses against HBV (Visvanathan et al., 2007; Wu et al., 2009). Along with these, the HBx protein when overexpressed can hinder the processing and presentation of viral antigens (Wieland and Chisari, 2005). These attributes of the virus contribute to viral persistence and provide tolerance against effector immune response.

The outcome of infection is determined in part by the age at which an individual is infected with HBV. Acute infection with HBV is characterised by a long incubation period in which the virus undergoes active replication before effector host immune responses produce a marked decline in virus levels. Clinical symptoms (malaise, anorexia, nausea, pruritus, jaundice) signs and abnormal investigations (raised levels of alanine aminotransferase, cell mediated immune responses) can be observed at between 1 and 6 months after infection (Dunn et al., 2009; Guidotti et al., 1996; Webster and Bertoletti, 2002; Webster et al., 2000). 1-5% of perinatal infections, around 50% childhood infections and more than 95% adult are cleared spontaneously within a period of 6 months since the time of infection, resulting in loss of

HBsAg and leading to lifelong immunity in most cases (Liaw and Chu, 2009). This is because an effective immune response is able to contain the virus rather than completely eradicate it (Rehermann et al., 1996). In instances of immunosuppression, the virus has the potential to reactivate.

Inability to resolve the infection leads to CHB. The natural history of CHB can be generally regarded to comprise five phases, which may not be experienced sequentially (2012) : the immune tolerant phase; the immune reactive HBeAg-positive phase; the inactive HBV carrier phase; HBeAg-negative CHB; and the HBsAg-negative phase. The immunotolerant phase is more frequent and long-lasting in individuals infected perinatally or in early childhood compared to those infected in adulthood. This phase is characterised by serum HBeAg positivity, high levels of HBV DNA, normal ALT. The immune reactive phase usually occurs after many years of immune tolerance in perinatally infected individuals, however, this stage is more rapidly reached in individuals infected in adulthood. It is characterised with decreased levels of HBV DNA, increased levels of ALT and a more rapid progression of fibrosis compared to the previous phase. This stage is terminated by reduction of HBeAg and seroconversion with the production of detectable levels of anti-HBe antibodies. (Dandri and Locarnini, 2012). Most frequently seroconversion to eAg negative CHB is followed by an inactive HBV carrier stage. This phase is charachterised by low (<2000 IU/ml) or undetectable levels of HBV DNA and normal ALT levels (EASL, 2012). The majority of patients in this phase for a long period of time have low risk of developing HCC or cirrhosis (Chen et al., 2012; 2012; Tai et al., 2009). HBeAg negative CHB is thought to be caused

by late immune reactivity to eAg negative variants of HBV and is characterised by persistently elevated or fluctuating levels of viral load and ALT (Papatheodoridis et al., 2008a). It may follow serconversion to anti-HBe at the end of immune reactive phase or it may develop years after the inactive HBV carrier state. The HBsAg-negative phase is characterised by the loss of HBsAg and may be accompanied by emergence of sAb. However low levels of viral replication may be detectable in the liver and not in the serum. Immunosuppression in these individuals may result in reactivation of HBV (EASL, 2012). If this phase is reached before the onset of cirrhosis, it is associated with a reduced risk of developing HCC and cirrhosis and decompensation.

These phases are useful in the clinical stratification of patients, however, they do not entirely define the complexity of immune responses during these stages. The innate and adaptive immune response, play an important role in resolving acute infection.

Innate immune responses

The liver is rich in cells that have the capacity to detect pathogens via pattern recognition receptors (PRRs). These include the hepatocytes that make up most of the liver parenchyma, the non-parenchymal cells (NPCs): liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs) and bone marrow-derived liver immune cells such as resident macrophages, kupffer cells (KC) and resident dendritic cells (DCs) (Protzer et al., 2012). Prototypic early responses to infection are characterised by the production of type I interferons (IFNs). However, in intrahepatic gene expression studies conducted in the chimpanzee model, in the early phases of HBV infection (entry and expansion) there were no detectable interferon-inducible genes (Wieland et al., 2004). Studies conducted with blood samples from patients with acute HBV infection confirmed these finding, as very low levels of circulating type I IFNs were detected (Dunn et al., 2009). Unlike infections with other viruses, such as human immunodeficiency virus (HIV), dengue virus and human cytomegalovirus (HCMV), acute infection with HBV often has a paucity of typical 'flu-like' symptoms and defective production of type I IFNs. HBV is often referred to as the 'stealth' virus owing to its capacity to evade host innate responses (Wieland and Chisari, 2005).

Studies conducted in the woodchuck model and virus-replicating cell lines have demonstrated that the levels of viral replication, size of viral inoculum and counteractive properties of NPCs influence the host innate immune responses and aftermath of infection (Asabe et al., 2009; Bertoletti et al., 2010; Guy et al., 2008; Lucifora et al., 2010). Gene studies in chimeric HBV

mice have shown that hepatocytes transiently activate the IFN-α producing gene upon infection, in line with the emerging concept that innate immune responses to the virus might be rapidly down-regulated (Lutgehetmann et al., 2011).

Type III IFNs that are produced primarily in the liver have also been shown to have anti-HBV activity in murine model, which may compensate for the lack of early type I IFN activity (Bertoletti et al., 2010).

HBV is a non-cytopathic virus. Liver injury and disease progression are attributed to host immune responses.

Natural killer T (NKT) cells and natural killer (NK) cells form an important arm of innate immune response. NKT cells are a heterogeneous population of cells that express markers of NK cells along with a T cell receptor, that recognise antigens presented by non-classical MHC class I like molecule CD1 (Seino and Taniguchi, 2005). In mice, around 30% of the intrahepatic lymphocyte population consists of NKT cells, while in humans it is less than 1% (Gao et al., 2009). NKT cells are further classified into classical or invariant NKT (iNKT) cells (>95% of total NKT cell population in mice) and non-classical type II NKT cells. Early on in the course of infection, NKT cells have the capacity to produce cytokines that can activate NK cells, and cells of the adaptive immune response: T cells and B cells (Tupin et al., 2007). Studies using the HBV transgenic mouse model have demonstrated that *in vivo* activation of NKT cells leads to secretion of antiviral cytokines in the liver

and therefore they have the potential to control viral replication (Kakimi et al., 2000).

Another NKT cell-like cell, which exhibits some of the iNKT cells' functional features along with a limited TCR repertoire diversity is called the mucosal-associated invariant T (MAIT) cell (Martin et al., 2009). On stimulation they produce large quantities of pro-fibrotic cytokines TNF-α and IL17 (Dusseaux et al., 2011; Walker et al., 2012). It has now been demonstrated by Tang et al. that MAIT cells are highly enriched in the human liver environment; their role in CHB remains to be determined. (Tang et al., 2013).

NK cells are typically amongst the first to become activated in early acute infection and have the potential to exert anti-viral functions in HBV infection (Dunn et al., 2009; Fisicaro et al., 2009; Guy et al., 2008; Kakimi et al., 2000; Yang et al., 2002). The frequency of NK cells is much higher in the healthy liver (30-40%) compared to their frequency in the periphery (~10%) (Cooper et al., 2001). Phenotypic studies of NK cells have demonstrated that activatory receptors NKp30, NKp46 and NKG2C are upregulated on NK cells of CHB patients compared to patients infected with HCV and healthy individuals (Bonorino et al., 2009; Rehermann, 2013). NK cells can exert antiviral effects through both cytolytic and non-cytolytic functions. Our group has shown that NK cells have the capacity to kill HBV infected hepatocytes and contribute to viral clearance along with causing liver injury in a TNF-related apoptosis inducing ligand (TRAIL) dependent manner (Dunn et al., 2007). NK cells also kill virus-specific $CD8^+$ T cells via TRAIL and in this way

contribute to viral persistence (Peppa et al., 2013). The non-cytolytic effector function of NK cells which includes production of anti-viral cytokines such as IFNγ is dampened in both early acute (Dunn et al., 2009) and chronic HBV infection due to high levels of IL-10 and TGF-β in the liver environment; this defect has been shown to be restored by blocking these cytokines *in vitro* (Peppa et al., 2010). The contribution of NK cell TRAIL-mediated killing of stellate cells to their anti-fibrotic potential in CHB has been described in the results section; investigation of this forms one of the main objectives of this study.

An overview of NK cells has been described in further detail in a subsequent sub-section.

Adaptive immune response

Helper $CD4^+$ T cells and effector $CD8^+$ T cells have both been documented to have a strong response in acute HBV infection (Guidotti et al., 1999; Rehermann et al., 1995). The helper $CD4^+$ T cells play a crucial role in influencing the quality and magnitude of eventual $CD8^+$ T cell responses against the virus (Asabe et al., 2009). Murine studies have shown that if the $CD4^+$ T cells are primed in the liver microenvironment, they fail to differentiate into Th1 cells and produce Th1 cytokines and this failure is thought to contribute to liver's tolerogenic environment and may dampen responses against the virus (Knolle et al., 1999). In the setting of CHB, $CD4^+$ T cells have a narrower virus-specific repertoire and a reduced proliferative potential (Ferrari et al., 1990). The role and frequency of typical regulatory T cells (Tregs), classified as $CD4^+$ $CD25^{high}$ $FoxP3^+$ cells, in the context of CHB is controversial, with conflicting evidence concerning their role (Li et al., 2012; Manigold and Racanelli, 2007). The *in vitro* depletion of Tregs in CHB has shown to improve the function of virus-specific T cells, this has also been reported in resolved infection (Franzese et al., 2005; Peng et al., 2008).

$CD8^+$ T cells also have a major contribution towards the adaptive immune response to HBV. The crucial role of $CD8^+$ T cells in controlling the virus in acute infection has been demonstrated in the chimpanzee model (Thimme et al., 2003). In acute HBV infection, $CD8^+$ T can directly kill virally infected hepatocytes cytolyticaly and contribute to liver injury leading to inflammation. $CD8^+$ T cells also produce cytokines such as TNF-α and IFN-γ non-

cytolytically to clear infection, as demonstrated in animal models and in *in vitro* cultures (Guidotti et al., 1996; Phillips et al., 2010). However, in chronic viral infection, due to prolonged antigen exposure, virus-specific effector $CD8^+$ T cells are profoundly depleted and have impaired effector functions, showing phenotypic characteristics of 'exhaustion'. This was first described in murine model of chronic disease with lymphocytic choriomeningitis virus (LCMV) (Wherry et al., 2003). This phenomenon has also been described in CHB and it has been observed that T cells of patients that are unable to control HBV viral replication have reduced production of IFNγ and exhibit a poorer proliferative potential (Boni et al., 2007; Das et al., 2008). In CHB, the level of HBV viral load and the duration of exposure of T cells to antigen correlates with changes in the effector functions of T cells (Bertoletti and Ferrari, 2012; Webster et al., 2004) HBV DNA levels have been observed to be inversely proportional to intra-hepatic virus-specific $CD8^+$ T cells (Fisicaro et al., 2010). Extensive gene profiling of virus-specific T cells from CHB patients compared to T cells form HBV resolved patients has shown that the former have high levels of pro-apoptotic molecule of Bcl-2 family, Bim, implying that these exhausted virus-specific T cells are more prone to premature apoptosis (Lopes et al., 2008). Defective antigen presentation in the liver has been suggested to contribute to vulnerability to apoptosis and pre-mature elimination of virus-specific T cells (Maini and Schurich, 2010), as described in mouse models (Holz et al., 2008). In patients on anti-viral treatment (that can suppress viral replication but not have a marked effect on the production of sub-viral particles), it has been observed that there is only a transient recovery of virus-specific T cells suggesting that persistent exposure to HBV

antigens affects T cell function (Boni et al., 2003). The imbalance in co-stimulatory versus co-inhibitory signals also contributes to the diminishing functions of $CD8^+$ T and their progression towards exhaustion. Over the years, many co-inhibitory molecules such as programmed death-1 (PD-1), cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), 2B4 and T cell immunoglobulin mucin-3 (Tim-3) have been described to be upregulated on virus-specific $CD8^+$ T cells in CHB (Boni et al., 2007; Nebbia et al., 2012; Raziorrouh et al., 2010; Schurich et al., 2011). It is worth noting that the liver is subjected to repeated exposure to gastrointestinal antigens, and so a level of immune tolerance is essential if the liver is not to be overwhelmed by inflammatory responses to pathogens and other foreign antigens. The tolerogenic activation of T cells by hepatocytes and LSECs may contribute to their exhaustion and susceptibility to apoptosis (Protzer et al., 2012).

Along with T cell responses, protective humoral responses by B cells also play an important role in the course of HBV infection. Antibodies against HBsAg are neutralising and these are associated with life-long protection against HBV infection and reinfection in an individual (Rehermann and Nascimbeni, 2005). Production of antibodies against Envelope protein have also been correlated with temporal clearance of the virus (Alberti et al., 1978). In CHB, the protective humoral immune response is affected by the dampened cellular immune response. Murine studies have demonstrated that B cells are able to produce regulatory cytokines such as IL-10, which influence other cells of the immune system (Fillatreau et al., 2002; Mauri et al., 2003). In CHB, a distinct population of B cells referred to as regulatory B

cells (Bregs) that are characterised by their production of IL-10 have been described as suppressing virus-specific $CD8^+$ T cell responses (Das et al., 2012).

NK cells: Overview

NK cells are large granular lymphocytes that have traditionally been regarded as belonging to the innate arm of the immune system; they are able to kill target cells without priming and are not restricted by the MCH of the target cell (Kiessling et al., 1975; Lanier et al., 1986). However, advances in the field have put NK cells on the border between innate and adaptive immune responses, with accumulating evidence that they can mediate recall/"memory" responses and can utilize their receptors to respond in an antigen-specific manner (Cooper et al., 2009; Paust et al., 2010; Rolle et al., 2013) highlighting the multiple functions of NK cells including tumor surveillance and response against pathogens (Vivier et al., 2011).

Conventionally, NK cells are phenotypically classified by the absence of CD3 and expression of CD56, which is a neural cell adhesion molecule (NCAM) (Lanier et al., 1989). Murine NK cells do not express CD56 and can be classified by their expression of NKp46, which is expressed by all mammalian NK cells (Walzer et al., 2007). A minority of human NK cells express low levels of NKp46. The function of these is yet to be determined.

NK cells primarily develop in the bone marrow. Bone marrow-derived CD34+ hematopoietic stem cells differentiate into common lymphoid progenitor cells that give rise to NK cell precursors that further develop into immature NK cells, eventually becoming functional mature NK cells after acquiring activating and inhibitory receptors (Lanier, 2005). *In vitro* experiments have

demonstrated that hematopoietic stem cells are dependent on stromal factors such as IL-7, IL-2, IL-15, stromal cell factor and FLT3 ligand at least in the very early stages of development of NK cells (Di Santo, 2006). Knowledge of NK cell development pathways in humans is still incomplete. Recent studies have indicated that immune precursor cells that hold the potential to develop into NK cells can be found in secondary lymphoid tissue and other anatomical locations including the liver (Eissens et al., 2012; Male et al., 2010; Moroso et al., 2011). These NK cells could represent a separate lineage of NK cells or imply that bone marrow-derived peripheral NK cells are not fully mature. These questions remain to be answered. In humans, the most complete pathway of NK cell differentiation is attributed to the sequential expression of specific markers and functionality of NK cells, which is reported to take place in secondary lymphoid tissue (Freud et al., 2005; Sun and Lanier, 2011). The four stages of differentiation have been classified by the expression of CD34, CD117 and CD95; these include: stage 1 pro-NK (CD34+CD117−CD94−); stage 2, pre-NK, (CD34+CD117+CD94−); stage 3, immature NK, (CD34−CD117+CD94−); and stage 4 (CD34−CD117+/−CD94+) NK cells, corresponding to the $CD56^{bright}$ subset.

In circulating blood, mature NK cells make up 10% of the peripheral blood lymphocyte population (Caligiuri, 2008). In lymphoid and non-lymphoid organs including liver, lung, thymus and uterus; different subsets of NK cells can be found (Colucci et al., 2003). Conventionally, human NK cells can be classified into two subsets depending in their cell surface expression of CD56: $CD56^{bright}$ and $CD56^{dim}$ (Cooper et al., 2001). $CD56^{dim}$ NK cells are enriched in

peripheral blood, bone marrow and spleen; while the CD56 ^{bright} NK cells form the majority in secondary lymphoid tissue (Fehniger et al., 2003). Both CD56 ^{bright} and CD56 ^{dim} subsets have distinct and crucial roles in immune response. The CD56 ^{bright} subset of NK cells is conventionally regarded as the primary source of NK cell-derived cytokines such as IFN-γ, TNF-β, Il-10, IL-13 and GM-CSF (Cooper et al., 2001), although more recently is has been recognised that the CD56 ^{dim} subset can also produce cytokines particularly upon target cell recognition (Fauriat et al., 2010). The CD56 ^{bright} subset of NK cells lack the low affinity Fcγ receptor CD16 and cytotoxic proteins perforin and granzyme and they express high levels of IL-2 receptor α chain (CD25) and inhibitory receptor complex CD94/NKG2A. By contrast, the CD56^{dim} subset of NK cells express high levels of CD16 and contains large number of granules of perforin and granzyme, leading to their cytotoxic function (Cooper et al., 2001). CD16 binds to the Fc region of immunoglobulin, activating NK cells to cause lysis of target cells via perforin. This is referred to as antibody-dependent cellular cytotoxicity (ADCC). Advances in the field have revealed that mature CD56^{bright} NK cells further mature to the CD56^{dim} phenotype (Caligiuri, 2008; Huntington et al., 2009; Romagnani et al., 2007).

NK cells express a wide array of activating and inhibitory receptors. The natural killer receptors (NKRs) can be classified into three categories; these are: the killer cell immunoglobulin-like receptors (KIR) that recognise classical MHC Class-I molecules (HLA A, B and C); the C-type lectins that recognise non-classical MHC Class-I molecules and class-I like molecules; and thirdly, the natural cytotoxicity receptors (NCR). Along with NKRs, NK cells also

express death ligands such as Fas-L and TRAIL. Interaction of death ligands with their respective ligands on target cells does not initiate cytotoxicity via NK cell degranulation but instead leads to caspase-mediated apoptosis of target cells. Examples of NKRs within each category are listed below:

KIRs	C-type lectins	NCRs	Death ligands
KIR2DL1-3	NKG2D	NKp46	TRAIL
KIR2DL5A-B	NKG2A	NKp44	Fas-L
KIR2DS1-5	NKG2C	NKp30	
		NKp80	

Table 1-1: List of some NKRs (classified as KIRs, C-type lectins and NCRs) and death ligands.
In red are the inhibitory receptors expressed on NK cells and in black are the activatory receptors expressed on NK cells.

NK cells express a greater variety of known activatory receptors compared to inhibitory receptors. The downstream signaling of activating receptors is varied and is dependent on the receptor. Activating receptors CD16, NCRs and C-type lectin NKG2C/CD94 interact with immunoreceptor tyrosine based activating motif (ITAM) containing adapters such as FcRγ, DAP12 and CD3ζ that activate further downstream signaling (Vivier et al., 2004). Other receptors such as NKG2D from the C-type lectin family initiates signalling upon interaction with its ligand via the DNAX-activating protein of 10kD (DAP10) (Lanier, 2005). The inhibitory pathways of NK cells express one or more intra-cytoplasmic immunoreceptor tyrosine based inhibition motif (ITIMs)

that activate and signal through SH2 protein tyrosine phosphatases (Vivier et al., 2004). The requirement for multiple signaling pathways remains unclear but may highlight the importance of multiple functions of NK cells.

The functional consequence of NKRs is a result of both activating and inhibitory signals received from interaction with their respective ligands on the target cell. Autologous normal cells are protected from effector NK cell attack because they express self-MHC Class I that engage with the inhibitory receptors (KIRs and NKG2A/CD94) on NK cells. Once the inhibitory receptors are engaged, their signals override any activating signal, preventing NK cells from killing any MHC-Class I expressing cell (Bryceson et al., 2006). However, murine studies have demonstrated that NK cells from MHC Class I deficient mice are hypo-responsive, implying the role of this interaction in development of NK cells to their competent state (Liao et al., 1991). Inhibitory signaling through MHC Class I is crucial for this education or licensing of NK cells to develop and mature completely (Bryceson et al., 2006).

Organ specific NK cells have the ability to adapt to the local microenvironment to facilitate tissue-specific functions, as seen in uterine NK cells during placentation (Hanna et al., 2006). In the liver, NK cells constitute around 40% of the total hepatic lymphocyte population. During viral infections in the liver, a further accumulation of NK cells is observed (Salazar-Mather et al., 1998). The production of chemokines by liver resident cells may be crucial for the selective homing of NK cells during disease. NK cells in the liver have a characteristic repertoire and cytokine profile (Lassen et al., 2010). The

majority of NK cells in the liver belong to the CD56 ^{bright} subset of NK cells, unlike the dominant circulating subset of mature CD56 ^{dim} CD16+ NK cells, suggesting that hepatic NK cells may constitute a liver-specific lineage, in line with their distinct transcriptional profile (Paust et al., 2010; Peng et al., 2013; Shi et al., 2011; Takeda et al., 2005). Recent studies in the field have demonstrated that all stages of NK cells can be found in an adult human liver and that peripheral NK cells recruited to the liver can attain a liver-specific phenotype (Moroso et al., 2011). The tolerogenic environment of a normal healthy liver maintains a functionally hyporesponsive state of liver NK cells by initiating the release of IL-10 by KCs to dampen down the effector functions of NK cells (Tu et al., 2008). IL-10 induces the expression of the inhibitory receptor NKG2A in liver resident NK cells to regulate their function (Krueger et al., 2011). In the liver, the number of licensed NK cells expressing inhibitory receptors for MHC Class I is less than in the periphery (Norris et al., 2003). In addition, hepatocytes express low level of self MHC Class I (Daar et al., 1984). These factors may contribute to the functional tolerance of hepatic NK cells. However, inflammatory cytokines have the potential to activate NK cells and overcome their hyporesponsive status (Burt et al., 2009; Kim et al., 2005).

In the liver, NK cells are primarily found in the hepatic sinusoid as illustrated in figure 1.1, however they can migrate via the fenestrations in the LSECs into the space of Disse and liver parenchyma, establishing a direct contact with HSCs and hepatocytes (Krueger et al., 2011).

Intrahepatic NK cells of healthy adults do not express TRAIL, however, in CHB, they have a preferential enrichment of TRAIL expression on CD56 ^{bright} subset (Dunn et al., 2007; Ishiyama et al., 2006). In CHB, NK cells can kill infected hepatocytes in a TRAIL-dependent manner (Dunn et al., 2007). NK cells have also been shown to lyse murine and human HSCs via TRAIL (Glassner et al., 2012; Radaeva et al., 2006). However, this has never been shown in CHB.

Intrahepatic NK studies in humans have been limited owing to lack of availability of healthy liver tissue.

Liver Fibrosis

Liver fibrosis refers to the accumulation of extra cellular matrix as a result of liver injury. It is a dynamic reversible wound-healing response in which matrix deposition is accompanied by matrix degradation. In cases of acute and self-limiting injury the fibrosis may be cleared. However, in chronic injury, persistent accumulation of extra cellular matrix and insufficient tissue remodeling leads to the formation of scar tissue. In advanced stages of fibrosis, the liver has around six times more ECM rich in fibrillar collagen than a normal liver (depending on the etiology), along with reduced activity of certain matrix metalloproteinases (MMPs) that help remove ECM, owing to overexpression of tissue inhibitor of metalloproteinases (TIMPs) (Arthur, 2000; Bataller and Brenner, 2005). Clinically, liver fibrosis can be assessed histologically using liver biopsies. The two commonly used well-established scales are the Metavir (I-IV) and the Ishak score (I-VI). However liver biopsy is an invasive procedure that is not well tolerated by patients and yields a small sample of liver tissue that may not be representative of the pattern of fibrosis throughout the liver (sampling error). Examination and staging of the severity of liver fibrosis by a liver pathologist is somewhat subjective and may give rise to inter-observer variation. For these reasons there has been a systematic endeavor to identify reliable non-invasive tests for assessing liver fibrosis. Various non-invasive tests have been successfully reported, some of which measure biochemical markers of liver function such as the FibroTest while others look at molecules involved in the formation and degradation of liver fibrosis such as the Enhanced Liver Fibrosis (ELF™) test (Myers et al., 2003; Parkes et al., 2010; Rosenberg et al., 2004). Progression of liver fibrosis

over a span of 30-40 years leads to cirrhosis, which is one of the main causes of mortality in CHB. Cirrhosis is characterised by distortion of normal liver architecture, formation of nodules, with disruption of the vascular architecture of the liver resulting in portal hypertension leading to variceal bleeding and ascites and eventually liver failure (Mormone et al., 2011). While some of the complications of cirrhosis can be treated presently, the only effective available treatment for cirrhosis is liver transplant and the shortage of donors poses a problem. Many cells have been implicated in liver fibrosis, these include peri-portal myofibroblasts, bone marrow derived myofibroblasts and epithelial-mesenchymal-transition (EMT); however, studies have repeatedly demonstrated that hepatic stellate cells (HSC), the resident mesenchymal cells, are the main cellular mediators of fibrogenesis in the liver (Friedman, 2008b).

HSC are located in the Space of Disse in the liver in between the liver sinusoidal epithelium and hepatocytes. In a normal healthy liver, HSC exist in a quiescent state. Quiescent HSC are characterised by the presence of large lipid droplets in the cytoplasm that have retinoids, fatty acids and triglycerides stored in them (Tacke and Weiskirchen, 2012). Around 80% of human body's vitamin A is stored in quiescent HSC in the form of retinoid (Korner et al., 1989). The quiescent cells have an elongated nucleus, enormous golgi apparatus and rather robust rough endoplasmic reticulum (ER), however they have poorly developed small ER and trifling numbers of mitochondria, lysosomes and peroxisomes (Tacke and Weiskirchen, 2012). They contribute to hepatic development and also play a vital role in liver regeneration in

adults. HSC form a part of the progenitor cell niche of a normal regenerating liver (Roskams, 2006; Yin et al., 1999). Studies have shown increase in the expression of epimorphin, a mesenchymal morphogenic protein by quiescent HSC after partial hepactomy (Yoshino et al., 2006). HSC play an important role in hepatic development especially in the development of intrahepatic bile ducts (Libbrecht et al., 2002). They also have the potential to secrete and produce several hepatocyte mitogens including hepatocyte growth factor (HGF), epimorphin and pleiotrophin (Friedman, 2008a).

As a result of insults producing liver injury, the quiescent HSC become activated and, as mentioned before, are the dominant cell type contributing to liver fibrosis. The activation of HSC broadly comprises two phases: initiation and perpetuation, which eventually lead to resolution if the source of liver injury is removed and the liver recovers (figure 1.4). Initiation of activation of HSC is usually a consequence of changes in the surrounding liver environment. These include the secretion of paracrine stimulants in the liver milieu and exposure to by-products of apoptotic or necrotic hepatocytes (Friedman, 2008b; Henderson and Iredale, 2007). Initiation refers to the changes in gene expression and phenotype of HSC that makes them responsive to the stimuli created in the injured liver environment. The inflammatory cytokines and growth factors in the liver form an essential part of the changes in the liver microenvironment. As persistent inflammation almost always precedes fibrosis, these cytokines play a crucial role in activating HSC. Gene array studies in mouse models and humans have demonstrated that inflammatory cytokines such as IL-1β, IL-6, IL-10, IL-13, IFN-γ and TNF-α

all contribute to fibrogenic responses (Bataller and Brenner, 2005; Safadi et al., 2004; Shi et al., 1997; Streetz et al., 2003). Transforming growth factor β (TGF-β) is one of the first cytokines to be activated from its latent form by neighbouring liver cells such as LSECs and KCs (Friedman, 2000). Injured LSECs also promote the production of fibrogenic cellular fibronectin (Jarnagin et al., 1994). KCs upon activation during liver injury activate HSC by stimulating matrix synthesis, secreting fibrogenic cytokines and reactive oxygen species (ROS) (Friedman, 2008a).

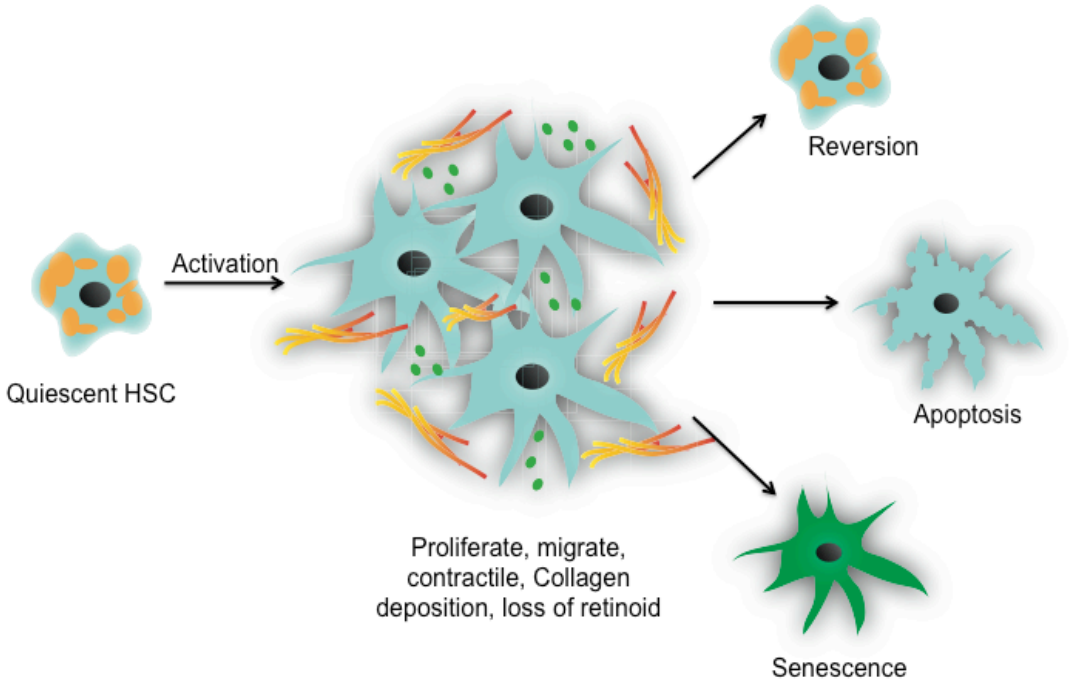


Figure 1-4: Fate of HSC.

During liver injury, the activation of HSC is initiated, and if the injury persists it perpetuates the activated state of HSCs making them contractile, promoting their proliferation and migration along with the deposition of collagen. If the cause of liver injury is removed, it leads to resolution of fibrosis by reversion, induction of apoptosis or senescence.

In the next phase referred to as the ‘perpetuation’ phase, the activated phenotype of HSC is maintained by these autocrine as well as paracrine changes, leading to their transformation into myofibroblast phenotype by an increase in their contractility, proliferation, migration towards chemoattractants, re-modeling of liver ECM, loss of retinoid and generation of fibrosis (Bataller and Brenner, 2005; Jiao et al., 2009). TGF-β and platelet-derived growth factor (PDGF) are the most extensively described fibrogenic and proliferative stimuli of HSC (Jiao et al., 2009). Other key growth factors and chemokines have also come to light. These include connective tissue growth factor (CTGF/CCN2) that functions via the TGF-β signaling pathway and is a potent fibrogenic agent and others such as insulin-like growth factor (IGF), epidermal growth factor (EGF) and chemokines such as CCL2 (also known as MCP-1) and CXCR3 that contributes to the chemotactic activity of HSC upon activation (Friedman, 2008a; Gao et al., 2004; Jiao et al., 2009; Marra et al., 1999). A characteristic feature of activated HSC is their increased expression of the cytoskeletal protein α-smooth muscle actin (α-SMA), which contributes to their contractile phenotype (Ramadori et al., 1990; Rockey et al., 1992).

Liver injury, which leads to HSC activation, can be caused via multiple mechanisms, such as reactive oxygen species (ROS), chemical toxicity and immune-mediated cytolysis, all of which may then trigger disruption of the homeostatic balance between MMPs and TIMPs. In fibrosis, this balance tips in favour of the TIMPs. Human studies have proven direct correlation between

the expression of TIMP-1 and -2 and the degree of fibrosis (Benyon et al., 1996).

In the context of chronic viral hepatitis B and C, liver fibrosis characteristically starts to develop around the portal tracts and progresses towards central lobular vein creating a portal-central bridge (Hernandez-Gea and Friedman, 2011; Pinzani, 1999). It has been demonstrated by treating rat HSC and LX2 with conditioned media from hepatitis B X protein (HBx)-expressing hepatocytes, that they lead to activation and proliferation of HSC (Martin-Vilchez et al., 2008). Histological longitudinal studies have shown that some CHB patients treated with NUCs have regression in cirrhosis (Hadziyannis et al., 2006; Marcellin et al., 2013). However, the mechanism behind these finding still remains to be investigated (Bedossa, 2015).

Role of Immune response in liver fibrosis:

In response to liver injury, both the innate and adaptive arms of the immune system become activated. Depending on the cause and duration of injury, some immune cells contribute to progression of fibrosis while others play an anti-fibrotic role. The pro- and anti-fibrotic roles of various immune cell types have been illustrated in Figure 1.5.

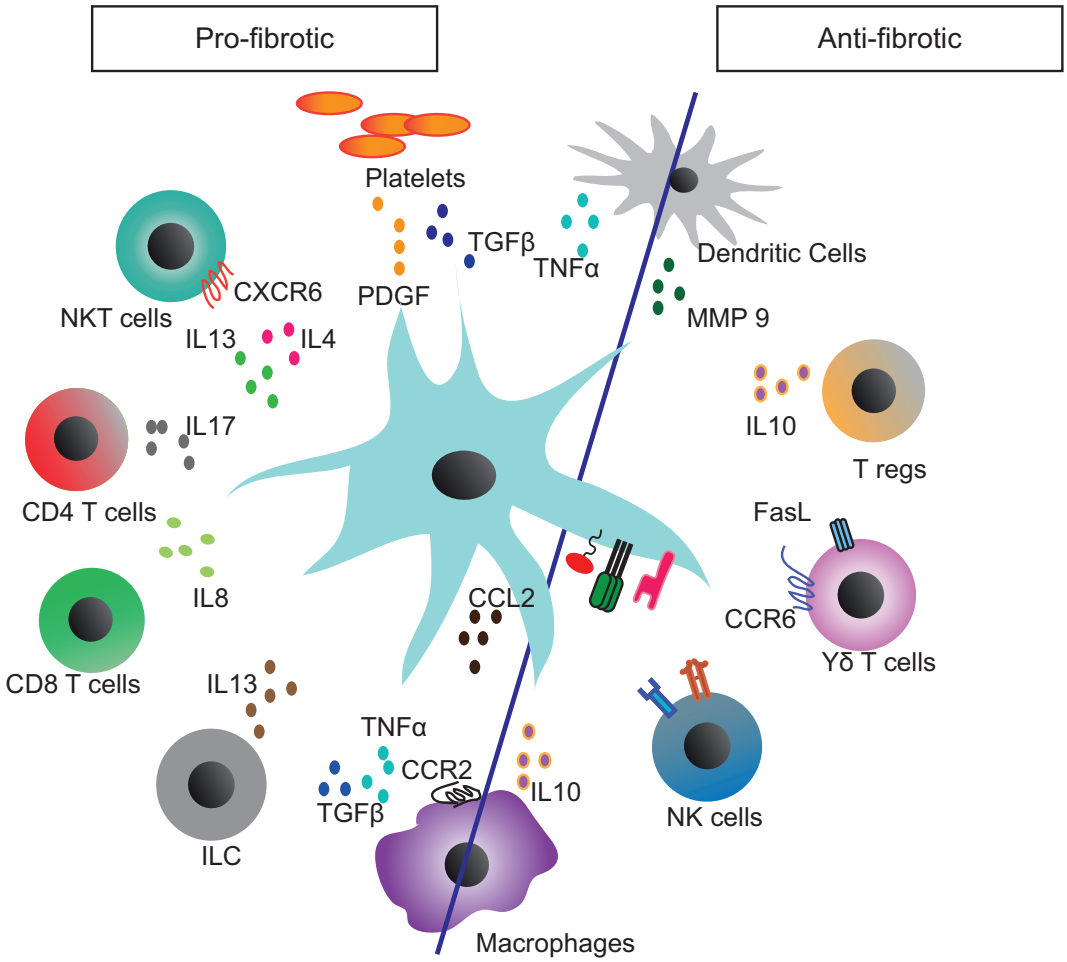


Figure 1-5: Immune cells and fibrosis.

The pro- and anti-fibrotic roles of various immune cells. Cells of the immune system that have been shown to have a pro-fibrotic effect include NKT cells, CD4 T cells, CD8 T cells, ILC and platelets. These cells produce cytokines and growth factors that activate HSC and perpetuate fibrosis. They also do so via ligand receptor interactions. Immune cells that have been shown to play an anti-fibrotic role are Tregs, γδT cells and NK cells. They do so by, either producing cytokines that prevent activation of HSC or by direct ligand-receptor interactions that lead to death of activated HSC. Macrophages and DCs can play both pro- and anti-fibrotic roles depending on which cytokine they produce.

The first cells to be recruited at the site of tissue damage are platelets. Along with their characteristic function of facilitating coagulation, they are the first cells to release PDGF and TGF-β, which are known mitogens and stimulants of HSC (Henderson and Iredale, 2007). The Guidotti group have used murine models to demonstrate that anti-platelet therapy is able to reduce the severity of fibrosis in HBV infection by reducing the capacity of platelets to drive an inflammatory infiltrate (Sitia et al., 2012; Sitia et al., 2013). However, a previous murine study has shown that in *in vitro* co-cultures, platelets are able to suppress the production of collagen-1 in HSC and the same study has demonstrated that thrombocytopenic mice have aggravated liver fibrosis, implying that platelets also have a potential anti-fibrotic role (Kodama et al., 2010). These discrepancies could be due to the use different mouse models to study two different liver diseases. Another immune cell type to infiltrate the site of injury early on is neutrophils. Casini et al. have demonstrated in co-culture systems that human HSC can be stimulated by reactive oxygen species derived from neutrophils (Casini et al., 1997). However, subsequent studies conducted in rats have shown that depletion of neutrophils did not have any effect on hepatic fibrogenesis following bile-duct ligation (BDL) (Henderson and Iredale, 2007; Saito et al., 2003). The complement system forms an important part of the host innate immune response not only as a defense for microbes but also viral infections and chronic inflammatory disease. The role of the complement in liver injury and fibrosis has been evident in several animal model studies. Work done using quantitative gene studies in chronic HCV in humans has implicated complement factor 5 (C5) in influencing liver fibrosis (Hillebrandt et al., 2005). High levels of C5aR1 have

been observed in murine HSC during differentiation into their activated myofibroblastic phase *in vitro* (Xu et al., 2012). Mice deficient in C5 have been shown to have an impaired potential for liver regeneration (Mastellos et al., 2001). In a cohort of one hundred and forty three CHB patients, in those with high ALT, serum complement 4 (C4) correlated with the degree of fibrosis established using a histological score (Bugdaci et al., 2011). The role of these various complement factors still remains to established in human livers.

Macrophages are bi-functional in the context of liver fibrosis. They have an important role to play during liver injury and progression of fibrosis as well as during the recovery or resolution of fibrosis. Persistent liver injury leads not only to the heavy infiltration of monocytes/macrophages, but also activation of the liver's resident macrophages, Kupffer cells, which make up the largest population of tissue resident macrophages in the human body. In animal studies, when monocyte/macrophage infiltration was suppressed during liver injury, the development of liver fibrosis was prevented (Ide et al., 2005; Imamura et al., 2005). Depletion of KCs from rat liver has been shown to attenuate chemical induced liver fibrosis, and this has been linked to reduced TGF-β in the liver environment (Rivera et al., 2001). Other groups have proven that depleting macrophages in advanced fibrosis reduced scar tissue, however, if they were depleted during recovery phase, the matrix failed to degrade (Duffield et al., 2005). Upon activation, macrophages and KCs are able to release multiple growth factors, cytokines and chemokines such as TGF-β, TNFα, IL8, CCL2 and CCL5 which are all highly pro-fibrogenic (Bataller and Brenner, 2005; Friedman, 2000; Henderson and Iredale, 2007;

Hernandez-Gea and Friedman, 2011). Macrophage derived lectin, Galectin-3 has been shown to play a role in both liver and kidney fibrosis by promoting activation to myofibroblasts (Henderson et al., 2006). Recent studies in the field have led to classification of macrophages into pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages (Laskin et al., 2011). In response to persistent injury, M1 macrophages contribute to inflammation and eventually fibrosis. Studies in HCV have emphasized the fibrogenic role of M1 macrophages and kupffer cells. These studies show that via HCV induced inflammasome signaling, macrophages produced IL-1β (Negash et al., 2013; Shrivastava et al., 2013). IL-1β promotes survival of activated HSC via the NF-κB signaling pathway. By contrast, in HBV transgenic mouse model, Sitia et al. found that kupffer cells can limit the severity of immunopathology in the liver without having any affect on the function of effector CD8+ T cells (Sitia et al., 2011). M2 macrophages have been shown to suppress fibrosis by producing the anti-inflammatory cytokine IL-10, and matrix metalloproteinase 13 (Fallowfield et al., 2007; Thomas et al., 2011).

In the past few years the lineage of innate immune cells has been studied more thoroughly. Along with conventional NK cells, other cell types have been classified on the basis of their transcription factors under the umbrella of innate lymphoid cells (ILCs). ILCs are branched into two lineages, the cytotoxic conventional NK cells and the other helper ILCs (ILC1, ILC2 and ILC3) (Diefenbach et al., 2014). A recent elegant study has implicated a role for ILC2 in mediating liver fibrosis. The study reports that during hepatocellular distress, IL-33 in secreted in the liver microenvironment, which

activates ILC2 and accumulates them in the liver; these in turn produce the profibrotic cytokine IL-13 leading to activation and transdifferentiation of HSC, contributing to liver fibrosis (McHedlidze et al., 2013). NK cells form a part of the cytotoxic ILCs.

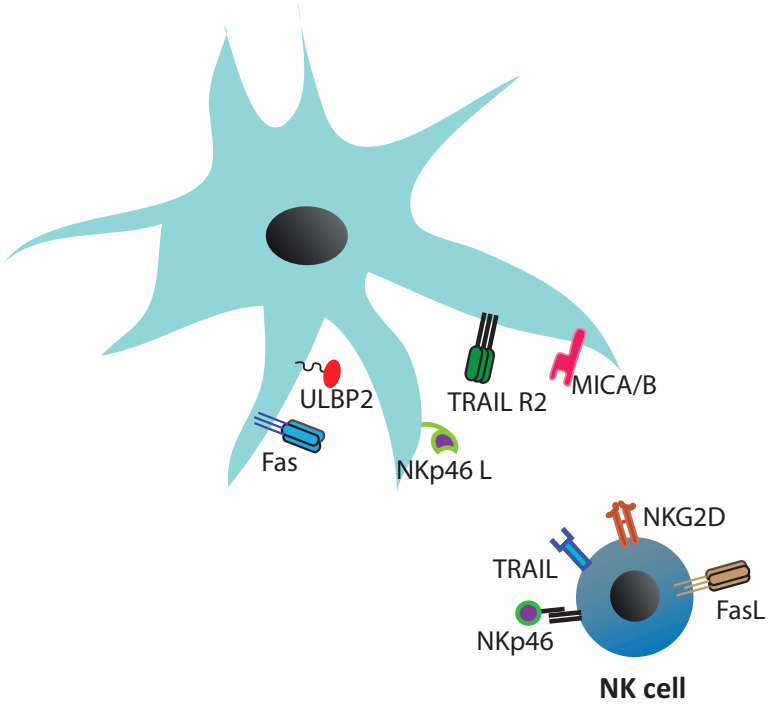


Figure 1-6: NK cell-HSC interactions.

Reported pathways via which NK cells can kill HSC. NK cells express death ligands TRAIL and FasL that can interact with their respective receptors, TRAIL-R2 and Fas on HSC and induce apoptosis of HSC. NK cells express the C-type lectin NKG2D that has been shown to activate NK cell cytotoxicity of HSC by interacting with their ligands ULBP-2 and MICA/B expressed on HSC. NKp46 expressed on the surface of NK cells has also been shown to drive NK cell cytotoxicity towards HSC. HSC have been shown to express the unknown ligand for NKp46 using fusion proteins.

Research in the field of NK cells in the context of liver fibrosis has gained interest in the past decade. NK cells have the capacity to kill hepatocytes in CHB (Dunn et al., 2007); through the induction of necroinflammation, they could contribute to the initiation of fibrosis. On the other hand, several groups have highlighted an anti-fibrotic role for hepatic NK cells. HSC express TRAIL receptor 2 and NKG2D ligands ULBP-2 and MICA/B (Glassner et al., 2012; Taimr et al., 2003). In animal models, it has been described that activated stellate cells downregulate MHC class I and upregulate TRAIL receptors, which renders them susceptible to killing by NK cells in a TRAIL and NKG2D-dependent manner (Melhem et al., 2006). When NK cells were depleted from these animals, severe progression of fibrosis was observed (Melhem et al., 2006; Radaeva et al., 2006). Recent studies performed by the Nattermann group have shown that NK cells from HCV infected patients are able to induce apoptosis of activated human stellate cells (Glassner et al., 2012). They have described that this is affected in a TRAIL, NKG2D and Fas-L-dependent manner (Glassner et al., 2012). The Safadi and the Mandelboim groups have together described that in mice, non-activated NK cells are able to kill HSC via NKp46, and on activation, other pathways contribute towards the killing of HSC. Even though the ligand for NKp46 is unknown, they have successfully shown that primary human HSC express the ligand using fusion proteins (Gur et al., 2012). There are no studies describing the role of NK cell mediated killing of HSC in HBV infection. The potential anti-fibrotic role of NK cells in CHB is one of the main focuses of this thesis and has been described in further details in the Results section. The various pathways via which NK cells can interact with HSCs are illustrated in figure 1.6.

NKT cells are a heterogeneous population of cells that express markers of NK cells along with a T cell receptor (Seino and Taniguchi, 2005). Studies conducted on animals by many groups over the years have described the important fibrotic role of NKT cells (Gao et al., 2009; Park et al., 2009). Jin et al. used an HBV transgenic mouse model to highlight their fibrogenic role via the secretion of IL-4 and IL-13 (Jin et al., 2011). Recent work done by Wehr et al. highlights the pro-fibrogenic role of NKT cells that is dependent on CXCR6 (Wehr et al., 2013). In human liver studies they observed upregulation of CXCR6 in the liver of patients with HCV infection compared to healthy controls. In mouse models they showed that CXCR6-deficient mice did not develop hepatic fibrosis (Wehr et al., 2013). Another NKT cell-like cell, the MAIT cell upon stimulation have been shown to produce large quantities of pro-fibrotic cytokines TNFα and IL17(Dusseaux et al., 2011; Martin et al., 2009; Walker et al., 2012). It has now been demonstrated by Tang et al. that MAIT cells are highly enriched in the human liver environment and are the pre-dominant cells (rather than CD4+ T cells) that produce IL-17A in the normal human liver (Tang et al., 2013). These studies implicate NKT cells in initiation and perpetuation of liver fibrosis.

Various groups have studied the role of T cells in liver fibrosis using different models. One of the most prominent studies done by Safadi et al. showed that the adoptive transfer of CD8+ T cells from carbon tetrachloride induced fibrotic livers of wild type mice into SCID mice, led to significantly increased levels of alanine aminotransferase (ALT) in the recipients and these rats

developed progressive fibrosis compared to those receiving CD4+ T cells or splenocytes (Safadi et al., 2004). This study highlighted a crucial role of CD8+ T cells in the pathogenesis of liver fibrosis. They also demonstrated that exposure to IL-10 can suppress the fibrotic activity of CD8+ T cells. Type 1 T helper cells (Th1) have been shown to have anti-fibrotic potential as they produce IFN-γ and IL-12 (Muhanna et al., 2008). Studies of Schistosoma mansoni infection in mice have emphasized the role of CD4+ T cells in liver fibrogenesis (Chiaramonte et al., 1999). In the same experimental model, studies have highlighted the pro-fibrogenic role of type 2 helper (Th2) CD4+ T cells by the production of IL-13 (Pellicoro et al., 2014). An imbalance between the anti-fibrotic Th1 cells and pro-fibrotic Th2 in chronic liver injury may influence fibrosis. Apart from these two types of CD4+ T cells, the crucial and potent pro-fibrogenic role of IL-17 producing CD4+ T cells (Th17 cells) has been established by animal and human studies on liver disease (Lemmers et al., 2009; Meng et al., 2012; Weaver et al., 2007). IL-17 has a direct effect on HSC and promotes production of collagen I. It can also have an indirect effect by interacting with all liver cells expressing IL-17R (KCs, monocytes, cholagiocytes) and lead to the production of pro-inflammatory cytokines (Pellicoro et al., 2014). Intrahepatic and peripheral Th17 CD4+ T cells are enriched in CHB and are associated with progression and severity of fibrosis (Sun et al., 2012). Tregs form another subset of CD4+ T cells and are classified by the expression of CD25 and transcription factor FoxP3. The number of Tregs is upregulated in livers of patients with viral hepatitis. In chronic HCV, the fibrotic role of IL-8 producing Tregs has been described (Langhans et al., 2013). Tregs also produce immunosuppressive cytokine IL-

10, which is known to inhibit activation of HSCs (Hammerich and Tacke, 2014). The Tacke group has recently described the anti-fibrotic role of γδT cells. γδT cells are recruited to the injured liver via the CCR6-CCL20 chemokine axis where they promote apoptosis of HSCs and have an anti-fibrotic role (Hammerich et al., 2014).

The role of B cells in liver fibrosis in the context of viral hepatitis is still not fully understood, however, studies in mice have shown that in a B cell deficient mouse model of liver fibrosis, there is reduced accumulation of collagen when compared to wild type mice (Novobrantseva et al., 2005). The development of liver fibrosis in this study was found to be antibody-independent.

Resolution of fibrosis

The attenuation or resolution of liver fibrosis is key to prevention of the complications of cirrhosis that characterize advanced chronic liver diseases including end-stage CHB. If this is to be achieved, it is crucial to understand the pathways via which the activation of HSC is resolved. Regression of liver fibrosis is associated with a decrease in the numbers of myofibroblasts from the site of scar tissue (Pellicoro et al., 2014). As described in figure 1.4, there are three possible fates of activated HSCs: reversion to quiescence, clearance through apoptosis and cell senescence. Removal of the cause of liver injury leads to changes in the liver microenvironment, which leads to resolution. In persistent injury, the levels of cytokines such as TGF-β are higher than normal and these promote the survival and activation of HSCs. Biochemical changes in the liver are essential to support regression of fibrosis. Reversion of activated HSCs to quiescence was thought to be possible only *in vitro* using basement membrane matrix. However, recent independent murine studies have demonstrated that downregulation of genes associated with fibrosis occurs in myofibroblastic HSCs which is similar yet distinct from typical naïve quiescent HSCs (Kisseleva et al., 2012; Troeger et al., 2012). These studies also demonstrated that these reverted HSCs have a higher susceptibility to activation upon treatment with fibrogenic stimuli.

Activated HSCs also express death receptors such as Fas and TRAIL-R2. Removal of pro-survival signals and engagement of these receptors with their respective ligands can induce apoptosis of activated HSCs. NK cells and intrahepatic γδT cells have the potential to induce apoptosis of HSCs and contribute to resolution of fibrosis. Another apoptotic pathway is the

overexpression of pro-apoptotic proteins such as B cell lymphoma 2 (BCL-2) and BCL-2 associated X protein (BAX) that can cause the apoptosis of HSCs via the caspase-9 route (Pellicoro et al., 2014). Since examination of apoptosis of HSC is one of the main objectives of this thesis, this pathway is described in greater detail in the next sub-section. The third potential pathway towards resolution of fibrosis is the senescence of myofibroblastic HSC characterised by their lack of cytokine synthesis and decreased ability to secrete MMP, eventually leading to their deletion by NK cells (Krizhanovsky et al., 2008).

Apoptosis

Apoptosis is a mechanism of carefully orchestrated programmed cell death that leads to dismantling of intracellular components of a cell in a controlled manner, avoiding inflammation and damage to surrounding cells. Apoptosis is essential for development and aging and plays a role in homeostatic maintenance of cell numbers in tissue (Elmore, 2007). There are multiple physiological and pathological stimuli that can trigger apoptosis, however, not all cells respond to the same apoptotic stimulus (Norbury and Hickson, 2001). Some cells require specific receptor-ligand interactions to undergo apoptosis while other cells have death pathways switched on by default that need to be blocked by strong survival factors such as hormones or growth factors (Elmore, 2007). There are two known apoptotic pathways: the external or death receptor pathway, and the intrinsic or mitochondrial pathway. These are illustrated in figure I7. The caspase cascade coordinates the process of apoptosis.

Caspases are primarily endoproteases that can hydrolyse peptide bonds (McIlwain et al., 2013). Mostly, caspases exist in an inactive monomeric state referred to as the procaspases that require to be dimerized and often cleaved for activation. They are assembled into dimers with the help of adapter proteins that can bind to specific regions of the procaspases (McIlwain et al., 2013). In humans, caspases are broadly classified on the basis of their function in apoptosis and in inflammation. The apoptosis causing caspases are further sub-classified into ‘initiator’ caspases that activate ‘executioner’ caspases leading to the demolition of structural proteins and DNA

fragmentation of the cell. Initiator caspases include caspase-8, -9, -10 and -2 that are produced as inactive procaspases. Upon dimerization they can catalyse cleavage of executioner caspase (caspase-3, -6 and -7) monomers into a large and a small subunit creating a stabilized functional mature dimer (Riedl and Shi, 2004). Upon activation a single executioner caspase has the capacity to activate other executioner caspases, creating a feedback loop of caspase activation.

The two apoptotic pathways can be differentiated on the basis of the adapters and initiator caspases involved. The extrinsic apoptotic pathway involves transmembrane receptor mediated interactions in the form of ligands binding to death receptors. Death receptors are members of the TNF superfamily and include TNF receptor-1 (TNFR-1), Fas, TRAIL-receptor 1 (TRAIL-R1) and TRAIL-receptor 2 (TRAIL-R2) (McIlwain et al., 2013). The death receptor ligands for these receptors are TNF, Fas-ligand (Fas-L) and TRAIL. When these ligands bind to their respective receptors, it leads to the recruitment of procaspase-8 to the site via its death domain (DD) to the death-inducing signaling complex (DISC) at the tail end of the engaged death receptor ligand (Grimm et al., 1996; Hsu et al., 1995). This interaction leads to the auto-catalytic activation of procaspase-8. Once caspase-8 is activated, it further activates executioner capase-3 (Walczak, 2013). Murine studies have demonstrated that mice deficient in caspase-8 are resistant to death receptor mediated apoptosis implying the crucial role of caspase-8 in the extrinsic apoptotic pathway (Juo et al., 1998).

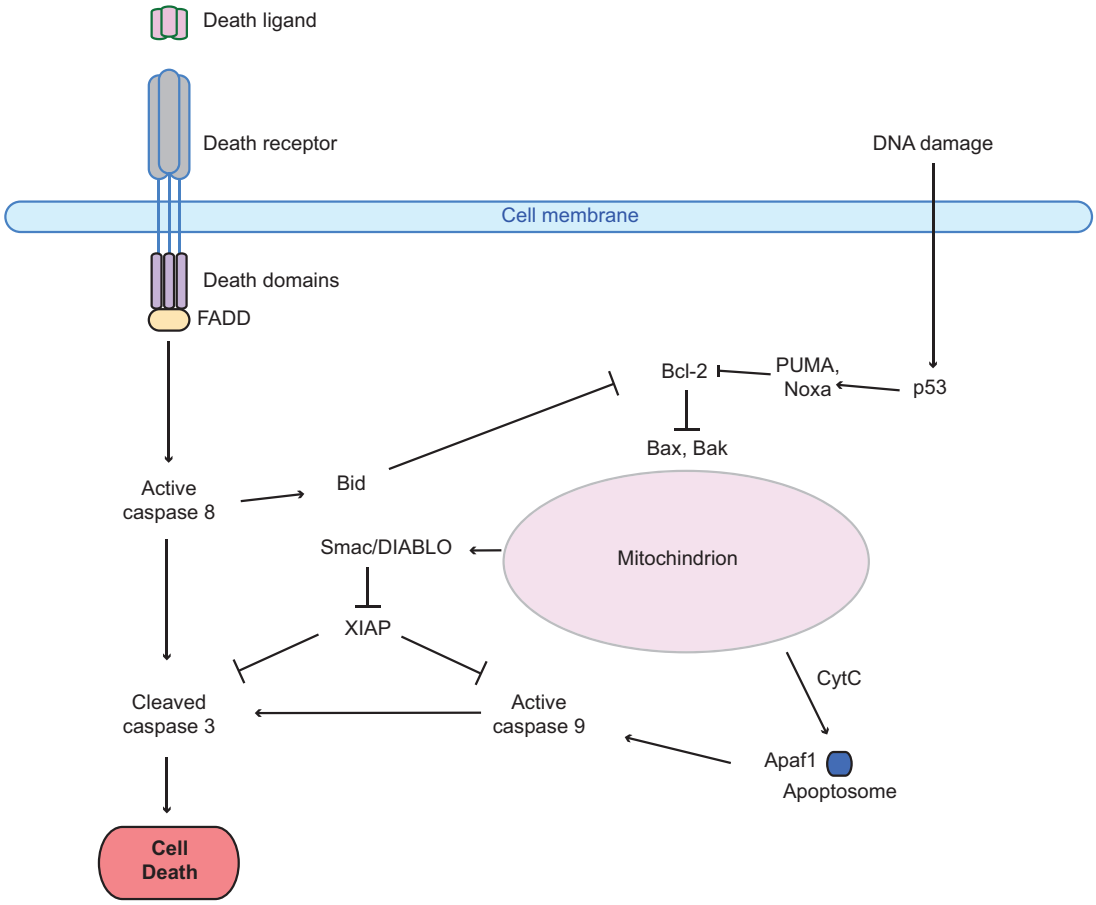


Figure 1-7: Extrinsic and intrinsic apoptotic pathways.

The extrinsic apoptosis pathway involves interaction of the transmembrane death receptor on target cells with its respective ligand. This leads to the recruitment of pro-caspase 8 at the site via the DD. Activation of caspase 8 leads to further downstream activation of caspase 3 leading to cell death. The intrinsic pathway is triggered by non-receptor-mediated stimuli that initiate mitochondrion associated caspase signaling. The mitochondrial transmembrane is permeabilised leading to the release of the pro-apoptotic molecules CytC, Smac/DIABLO into the cytoplasm, which leads to activation of caspase 9 which in turn activates caspase 3 leading to cell death.

The intrinsic apoptotic pathway is initiated by non-receptor-mediated stimuli that produce intracellular signals targeting the mitochondrion associated caspase signaling (Elmore, 2007). The intracellular signals initiated by these stimuli could either be positive signals such as hypoxia, toxins, radiation, free radicals etc. that induce apoptosis or negative signals such as lack of growth factors or hormones that suppress the cell's inherent death pathway, thereby triggering apoptosis. All these stimulations lead to the opening of mitochondrial permeability transition pores. The loss of the mitochondrial transmembrane facilitates the release of otherwise sequestered pro-apoptotic molecules cytochrome c, Smac/DIABLO and serine protease HTRA2/Omi into the cytosol (Du et al., 2000; Garrido et al., 2006; Saelens et al., 2004). Cytochrome c binds and activates procapase-9, while SMAC/DIABLO and HTRA2/Omi prevent the suppression of apoptosis by inhibitors of apoptosis proteins (IAP) (Hill et al., 2004; van Loo et al., 2002). Inhibiting IAP is not enough to initiate caspase, the second group of pro-apoptotic proteins are released from the mitochondria once the cell is committed to die; these include AIF, endonuclease G and CAD (Elmore, 2007). AIF and endonuclease G causes DNA fragmentation and chromatin condensation, while CAD is cleaved by activated caspase-3 to provide a further more pronounced second stage of chromatin condensation (Joza et al., 2001; Susin et al., 2000). The Bcl-2 family of proteins plays a crucial role in regulating mitochondrial events leading to apoptosis. The Bcl-2 family is in turn regulated by a tumour suppressor protein *p53* (Schuler and Green, 2001). The Bcl-2 family comprises both pro- and anti-apoptotic genes that regulate the intrinsic apoptotic pathway. The pro-apoptotic proteins include Bid, Bim, Bcl-10, Bik,

Bak, Bad and the anti-apoptotic proteins include Bcl-2, Bcl-X, Bcl-XL, Bcl-w, BAG (Elmore, 2007; Riedl and Shi, 2004). The main role of the Bcl-2 family of proteins is to manage the release of cytochrome c from the mitochondria by manipulating mitochorial membrane permeability.

Once the initiator caspases of both extrinsic and intrinsic pathways have cleaved and activated executioner caspases, the executioner caspases further activate cytoplasmic endonucleases and proteases, causing the degradation of vital cellular and nuclear proteins (McIlwain et al., 2013). Executioner caspases cleave cytokeratins, cytoskeletal proteins, poly ADP ribose polymerase (PARP), nuclear proteins and others (Slee et al., 2001). Caspase-3 is the most important executioner caspase and has a unique ability to activate CAD from ICAD leading to degradation of chromosomal DNA and persistent chromatin condensation (Sakahira et al., 1998). Caspase-3 also contributes to cytoskeletal disintegration by cleaving gelsolin, an actin-binding protein (Kothakota et al., 1997).

Phagocytic uptake of apoptotic cells is the final phase of apoptosis. In this phase, the cell externalizes phosphotidylserine on the surface of the apoptotic cell, facilitating non-inflammatory recognition of the cell, leading to early and efficient uptake without causing the spillage of any cellular components that may cause an inflammatory reaction (Fadok and Chimini, 2001).

In summary, the role of lymphocytes in eliciting an innate and adaptive immune response in CHB has been well documented, however, their role in CHB associated liver fibrosis still remains to be investigated. In CHB, NK cells may exert anti-viral effector function but can also delete virus-specific CD8 T cells to contribute to viral persistence. The anti-fibrotic role of NK cells has been established in animal models and other liver diseases that lead to fibrosis. Since NK cells have an altered phenotype and defective effector function in CHB, in this study we wanted to investigate their potential to limit HSC-mediated fibrosis in this context. We also investigate the pro-survival anti-apoptotic nature of activated HSC that maintains them in a fibrotic liver undergoing chronic injury.

Hypothesis:

Immune responses mounted in patients with CHB exert pro- or anti-fibrogenic effects on hepatic stellate cells, thereby potentially regulating liver fibrosis progression.

Specific aims:

1. To examine whether peripheral blood mononuclear cells from patients with CHB can produce soluble mediators that are capable of activating HSC.
2. To investigate whether NK cells from patients with CHB are able to induce apoptosis of activated HSC thereby exerting an anti-fibrogenic effect.
3. To examine the anti-apoptotic phenotype of HSC contributing to their escape from TRAIL mediated killing.

2. Materials and Methods

Patients and healthy controls

Local ethical boards at Camden Primary Care Trust, University College Hospital, Royal London Hospital and Royal Free Hospital Trust approved this study. All participants of the study have given written, informed consent. All sample and data storage complies with the requirements of Data Protection Act 1998 and Human Tissue Act 2004. Peripheral blood was obtained from patients infected with CHB attending clinics at Mortimer Market Centre (Bloomsbury, London), University College London Hospital NHS Foundation Trust (Bloomsbury, London), Royal London Hospital, Barts Health NHS Trust (Whitechapel, London) and Royal Free London NHS Foundation Trust (Hampstead, London). All HBV infected patients were anti-HCV and anti-HIV antibody negative. The patients were stratified on the basis of their disease status: viral load (determined by real-time PCR), liver inflammation indicated by serum levels of alanine transaminase (ALT) and degree of fibrosis determined by Ishak stage and/or ELF test (Ishak score: 1-2 mild fibrosis, 3-4 moderate fibrosis; ELF test: 0-7.7 no/mild fibrosis, 7.7-9.8 moderate to severe fibrosis). For the isolation of primary human HSC, liver tissue was obtained from healthy margins of non-HBV metastatic livers. The clinical details of the patients and the healthy controls that participated in this study are given in subsequent Results chapters.

Isolation of peripheral blood mononuclear cells

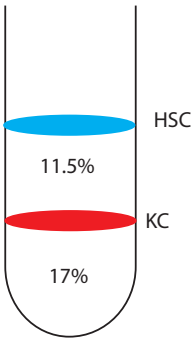
Up to 50ml of venous blood was collected from all consented donors in 10ml BD Vaccutainer® containing EDTA or sodium heparin and processed immediately. The peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-plaque Plus (GE Healthcare, Buckinghamshire, UK) density gradient centrifugation. In a 50ml falcon tube, 20ml of whole blood was carefully layered on top of 15ml of Ficoll-Plaque Plus and centrifuged at 2200 rpm at 20°C for 22 minutes without brakes. The PBMC were carefully aspirated from the interface using 2 ml Pasteur pipettes and diluted 1:1 with RPMI 1640 (Invitrogen™, California, USA) and washed at 1800 rpm at 20°C for 15 minutes. The PBMC pellet was re-suspended in RPMI 1640. The cells were diluted 1:9 in trypan blue (Sigma-Aldrich, St Louis, USA) and were counted using Neubauer counting chamber under a light microscope. The number of cells was determined using the following formula:

$$\text{Number of cells/ml} = \text{number of cells counted} \times \text{dilution factor} \times 10^4$$

The cells were washed and re-suspended in foetal bovine serum (FBS) (Invitrogen™, California, USA) with 10% DMSO (Sigma-Aldrich, St Louis, USA) at a concentration of 10^7 cells/ml and transferred into cryovials (Corning, New York, USA). Cryovials were frozen at -80°C in Mr Frosty® (Fisher Scientific, Waltham, USA) for up to 24 hrs and then transferred to gas-phase nitrogen storage tanks.

Isolation of primary human hepatic stellate cells

Healthy margins of metastatic liver tissue were collected from consented patients during surgery. The tissue was washed in PBS and transferred to a petri plate (Sarstedt, Numbrecht, Germany) containing a PF3 buffer made up of Hank's balanced salt solution (HBSS) (Invitrogen™, California, USA) with calcium and magnesium, 0.01% collagenase IV and 0.001% DNAse I. The tissue was then passed through tissue press (2mm diameter holes) and was collected into a 50ml falcon tube (BD Falcon, Oxford, UK) containing 10 ml of the PF3 buffer and incubated at 37°C water bath for 20 minutes with occasional shaking. The homogenate was filtered through a 70 µm cell strainer (BD Falcon, Oxford, UK) into a new falcon tube using the back of a 10ml plunger and centrifuged at 500 rpm for 2 minutes at 4°C to get rid of the hepatocytes. The supernatant was transferred in to a new falcon tube and centrifuged at 1500rpm for 10 minutes at 4°C. the pellet comprising of non-parenchymal liver cels was re-suspended in PF4 buffer made up of HBSS without calcium and magnesium containing 0.001% DNAse I and 0.25% BSA. The pellet was re-suspended in 10 ml of 17% Optiprep® (density gradient diluted in PF4) (Sigma-Aldrich, St Louis, USA). 10 ml of 11.5% of Optiprep® was carefully layered on top of it. Additional 4ml of PF4 was layered on top of it and centrifuged at 2700 rpm for 17 minutes at 20°C without any breaks. The hepatic stellate cell fraction on top of the 11.5% optiprep layer was aspirated using a 2ml Pasteur pipette.



An equal volume of PF4 was added to it and centrifuged at 1500 rpm for 10 minutes. The pellet was re-suspended in SteCM media (ScienCell Research Laboratories, California, USA) and counted as described previously. The cells were plated at a density of 5 x 10^4 cells/cm^2 in appropriate sized tissue culture flasks (NUNC, Waltham, USA). It is noteworthy that at this stage, freshly isolated HSC need to be cultured in the tissue culture flasks instead of tissue culture plates. It was observed while optimising this protocol that freshly isolated HSC plated on tissue culture plates did not proliferate or grow very well on plates; as a result they could not be expanded and frozen for future use. Plausible reasons for this could be the difference in the plastic of the tissue culture flask and tissue culture plates, secondly the surface area available for cells to grow is much less than in a flask which may influence their growth and proliferative potential. The cells were left for 24 hours to attach and were then washed with media and fresh media was added. The quiescent hepatic stellate cells started differentiating into an activated state in around 7-10 days post culture. The cells were then passaged twice before freezing them down using FBS and 10% DMSO as previously described.

Isolation of intrahepatic lymphocytes

In a petri-plate (Sarstedt, Numbrecht, Germany), liver tissue was suspended in RPMI 1640 (Sigma Aldrich) and using mechanical force, macerated using back of a 10 ml syringe plunger. The cell suspension was then passed through 70mm cell strainer (BD Biosciences) several times and then centrifuged at 1800 rpm for 15 minutes to get rid of cell debris. The pellet was resuspended in RPMI 1640 (Sigma Aldrich) supplemented with 10% foetal bovine serum (Invitrogen™, California, USA)) for counting. The cells were then used for subsequent experiments.

Isolation of NK cells using magnetic beads

NK cells were isolated from PBMC by negative selection using CD3 magnetic beads and then by positive selection using CD56 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) or by using NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instructions. The purity of NK cells was checked using flowcytometry by gating on CD56+ and CD3- cells. The purity of NK cells obtained was always greater than 95%. No difference was found in the quality or purity of NK cells when either positive or negative selection was used. It is noteworthy that in some assays positive selection may activate the cells as the magnetic microbeads directly bind to the NK cells. However, this was not the case in assays done in this thesis. In the majority of experiments negative selection was performed using the NK cell isolation kit as it is faster and takes half the time compared to positive isolation.

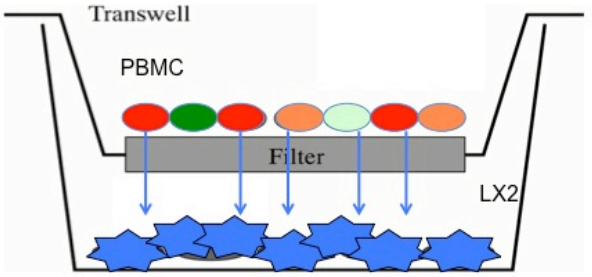
Culturing LX2 cell line

LX2 is an immortalized HSC cell line isolated in Professor Scott Friedman's lab. This cell line was used to optimise and perform experiments. LX2 cell line expresses most markers of activation of HSC, such as high levels of αSMA, Collagen 1, vimentin etc (Xu et al., 2005). Even though this is an activated cell line, it has been well established in our lab (Rosenberg lab) and others that LX2 cells further up-regulate activation markers in response to stimulation. LX2 cells were defrosted and cultured in tissue culture flasks in media comprising of DMEM (Invitrogen™, California, USA) supplemented with 10% FBS (Invitrogen™, California, USA). When cells reached 80% confluence, they were passaged and plated in 48-well or 24-well plates in 300µl and 500µl of DMEM+10%FBS respectively for subsequent experiments.

Experiments using a transwell system

Cryopreserved PBMC were thawed and counted. The cells were re-suspended in RPMI 1640 supplemented with 10% FBS, 100U/ml penicillin/streptomycin (Invitrogen™, California, USA), MEM essential amino acids (Invitrogen™, California, USA), MEM non-essential amino acids (Invitrogen™, California, USA), HEPES (Invitrogen™, California, USA), β-mercaptoethanol (Sigma-Aldrich) and sodium pyruvate (CRPMI, Invitrogen™, California, USA). The cells were plated in 96 U-bottomed plates at 30 x 10^5 cells/well in 200µl volume with medium only (negative control), 1µM HBV overlapping (OLP) peptide which is a pool of 15mer peptides overlapping by 10 residues spanning 24 peptides of the core of HBV (genotype D, JPT), or 0.5µM PepMix CEF Pool (32 peptides from CMV, EBV, Influenza virus, JPT as a positive control) with rIL-2 (Roche) supplement (20U/ml). Half the media

was replaced with fresh CRPMI supplemented with rIL-2 on day 3 of culture. Two parallel plates were setup for each experiment.



The PBMC were restimulated with relevant peptides and cultured on transwell membranes (0.3µm) on day 9 of culture with LX2 cells (plated on 24-well transwell plates, Beckton Dickinson, New Jersey, USA 24 hours prior). 16 hours post culture in the transwell system, supernatant from one of the plates was stored in -20°C freezer for future Real time PCR experiments. 24 hours after transwell system culture, LX2 cells from the second plate were harvested and stained (surface and intracellularly) for markers of activation. The cells were acquired on BD^{TM} LSR II flow cytometer (Beckton Dickinson) and analysed using FlowJo (Treestar).

Real time PCR to examine the activation of LX2

Stored supernatant from the transwell experiment were applied to LX2 cells (plated on 48 well plate 24 hour prior) for 16 hours. The LX2 were harvested, lysed and their RNA was extracted using the TaqMan® Gene Expression Cells-To-CTTM kit (Applied Biosystems, Paisley, UK) according to manufacturer's instructions. The lysates were converted to cDNA using

reverse transcription according to manufacturer's instructions. For running the Real time PCR, the gene expression assays used were αSMA and Pro-Collagen I (ACTA2, COL1, Applied Biosystems Paisley, UK). The house-keeping gene used was GAPDH (GAPDH, Applied Biosystems Paisley, UK). All the gene expression assays were labeled with FAM. The PCR cocktail was added on to 384-well PCR plates to which the cDNA was added in triplicates. The PCR was run on Applied Biosystems 7900 HT machine. The data were analysed using -2ΔΔCT method as described Livak et al. (Livak and Schmittgen, 2001).

Apoptosis assay

Cryopreserved primary HSC were thawed and cultured in T-25 tissue culture flask (NUNC, Waltham, USA) for 4 days at 37ºC with 5% CO_2. The primary HSC were then detached using 0.05% Trypsin/EDTA (Invitrogen™, California, USA) and plated on 48 well plates (Corning, New York, USA).

Isolated NK cells and primary HSC were co-cultured at an E:T ratio of 10:1 for 5 hours in SteCM. After co-incubation, the primary HSC were harvested and their apoptosis was studied by intracellularly staining for active caspase-3 using active caspase-3 apoptosis kit (BD Bioscience, Oxford, UK) according to manufacturer's instructions. The data were collected on BD™ LSR II flow cytometer (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar, Oregon, USA). The positive control for apoptosis experiments was 500ng/ml of *SuperKiller* TRAIL (Enzo Lifesciences, Exeter, UK) which is a trimeric stable form of recombinant TRAIL and induces apoptosis of cells via

the Caspase 3 pathway.

Blocking experiment

TRAIL-R3 (MAB-630, R & D Systems, Minneapolis, USA) and TRAIL-R4 (MAB-633, R & D Systems, Minneapolis, USA) neutralising antibodies were used to block these receptors on primary HSCs. The antibodies were added at a concentration of 5 µg/ml for 30 minutes to HSC cultures. The neutralising antibodies were washed off using PBS prior to setting up apoptosis assays.

Real time PCR for baseline mRNA levels of αSMA and TRAIL-receptors on LX2 and primary HSC

LX2 and primary HSCs were lysed and their RNA was extracted using the TaqMan® Gene Expression Cells-To-CT^{TM} kit (Applied Biosystems Paisley, UK) according to manufacture's instructions. The lysates were converted to cDNA using reverse transcription according to manufacturer's instructions. For running the Real time PCR, the gene expression assays used were αSMA, TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 (Applied Biosystems Paisley, UK). The house-keeping gene used was GAPDH (GAPDH, Applied Biosystems Paisley, UK). All the gene expression assays were labeled with FAM. The PCR cocktail was added on to 96-well PCR plates to which the cDNA was added in triplicates. The PCR was run on Applied Biosystems 7900 HT machine. The data were analysed using $-2^{\Delta\Delta CT}$ method as described Livak et al. (Livak and Schmittgen, 2001).

Flow cytometry

All stains for flow cytometry analysis were performed on single cell suspensions in 96 U-bottomed plates (Sarstedt, Numbrecht, Germany) at 4ºC.

Surface staining of PBMC/pHSC/LX2

Following cell culture, cells were washed in 1x PBS (Invitrogen™, California, USA) and centrifuged at 1600rpm for 6 minutes. The supernatant was discarded and the pellets of cell were re-suspended by gentle vortexing. The cells were stained with Live/Dead® Cell viability stain (Invitrogen™, California, USA) and the relevant anti-human antibodies in 1x PBS for 30 minutes at 4ºC in the dark. Appropriate isotype controls were used where necessary. Post incubation the cells were washed and fixed with 150 µl/well of BD™ Cytofix. Cells were transferred to 500 **µ**l polystyrene tubes for acquisition. Data was acquired on BD™ LSR II flow cytometer (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar Oregon, USA).

Surface staining of intrahepatic cells isolated from liver explants and biopsies

Cells were washed in 1x PBS (Invitrogen™, California, USA) and centrifuged at 1600rpm for 6 minutes. The supernatant was discarded and the pellets of cell were re-suspended by gentle vortexing. FcR blocking reagent (Miltenyi) was added to the cells to avoid non-specific binding of antibodies at a concentration of 1:5 for 10 minutes. Live/Dead® Cell viability stain (Invitrogen™, California, USA) and the relevant anti-human antibodies in 1x PBS were then added to the cells without washing for 30 minutes at 4ºC in the

dark. Appropriate isotype controls were used where necessary. Post incubation the cells were washed and fixed with 150 µl/well of BD™ Cytofix (Beckton Dickinson, Oxford, UK). Cells were transferred to 500 µl polystyrene tubes for acquisition. Data was acquired on BD™ LSR II flow cytometer (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar Oregon, USA).

Intracellular staining of PBMC/pHSC/LX2

Cells were surface stained as described above, following which they were washed and re-suspended in BD™ Cytofix-Cytoperm (Beckton Dickinson, Oxford, UK) for 20 minutes at 4ºC in the dark. After permeabilisation and fixation the cells were stained intracellularly using the relevant anti-human antibodies in 0.1% saponin for 30 minutes at 4ºC in the dark. Appropriate isotype controls were used where necessary. After staining the cells were washed and re-suspended in 150 µl/well of BD™ Cytofix (Beckton Dickinson, Oxford, UK). Data was acquired on BD™ LSR II flow cytometer (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar).

The antibodies used for both extra- and intra-cellular staining are listed below in table 3.

Marker	Fluorochrome	IgG	Manufacturer
Live/Dead® stain	Blue		Invitrogen
CD3	PE Cy7	$IgG1_{\kappa}$	eBiosciences
TRAIL	PE	IgG1	eBiosciences
CD56	ECD	IgG1	Beckman Coulter
NKG2A	Alexa Fluor 700	IgG2A	R&D Systems
NKp46	BD v450	$IgG1_{\kappa}$	BD Biosystems
HLA DR	BD v500	IgG2A	BD Biosystems
NKp30	APC	IgG1	Miltenyi
CD16	APC Cy7	IgG1	BD Biosystems
Caspase 3	PE	IgG	BD Biosystems
TRAIL-R1	PE	IgG1	R&D Systems
TRAIL-R2	PE	IgG2B	R&D Systems
TRAIL-R3	PE	IgG1	R&D Systems
TRAIL-R4	PE	IgG1	R&D Systems
αSMA	PE	IgG_{2A}	R & D Systems
CD19	APC Cy7	IgG1	Biolegend
CD68	FITC	IgG1	eBiosciences
Cytokertain	FITC	IgG1	eBiosciences
CD14	BD v500	IgG1	BD Biosystems
CCL2	FITC	IgG_{2B}	R & D Systems
ICAM1	Pacific Blue	$IgG1_{\kappa}$	Biolegend
PDGFRβ	APC	$IgG1_{\kappa}$	Biolegend
αSMA	PE	IgG_{2A}	R & D Systems

Table 2-1: List of antibodies for flow cytometry

Directly conjugated anti-human flow cytometry antibodies extra- and intra-cellular antigens with respective isotypes.

Transducing HSC using lentiviral vectors with short hairpin

The lentivector pSIN-GFP was digested with EcoRI and BamHI, and the promoter of the gene encoding phosphoglycerate kinase (PGK) was amplified by polymerase chain reaction (PCR) for introduction of EcoRI-BamHI restriction sites. Then, the SFFV promoter from the plasmid pSIN-GFP was replaced with the promoter of the gene encoding PGK, cloned by blunt-end ligation, to generate the plasmid pSIN-PGK-GFP. A second cassette containing the U6 promoter was cloned in this backbone downstream the cPPT sequence between ClaI-EcoRI restriction sites. A BamHI site was introduced downstream of the U6 promoter to clone the short hairpin RNA (shRNA) of interest between BamHI and EcoRI restriction sites. The U6-shRNA cassette was placed upstream the PGK promoter and GFP. All constructs were engineered by standard cloning techniques.

The following shRNA sequences were used:

shTR2:

CTCACTGGAATGACCTCCTTTCTCGAGAAAGGAGGTCATTCCAGTGAG

shTR4:

GATGGTCAAGGTCAGTAATTGTTCAAGAGACAATTACTGACCTTGACCATCTT

TTTTACGCGT

shCTR:

CCTAAGGTTAAGTCGCCCTCGTTCAAGAGACGAGGGCGACTTAACCTTA

GG

Knockdown progress was observed by monitoring GFP expression using fluorescence microscopy. Knockdown lentiviruses were produced using a packaging cell line (293T-cells) and transfected with p8.91 (encoding structural proteins, gag, pol, rev, tat), pMDG (encoding VSVg env) and transfer vectors pSIREN shCTR or pSIREN shTR2. This work was done in collaboration with Itziar Otano (postdoc, Maini Lab).

The shCTR and shTR2 carrying lentivirus were transduced into HSC plated in a 24 well plate in 500µl volume of SteCM at an MOI (multiplicity of infection) of 40 for upto three days. Transduction was checked microscopically to look for GFP+ cells. Transduction and knockdown was confirmed using FACS. The cells were then used for subsequent experiments.

3. PBMC from CHB patients produce soluble mediators that activate stellate cells

Background:

Liver fibrosis is a common consequence of CHB. As mentioned in detail previously, HSC activated by changes in the liver milieu are the main cellular source of hepatic fibrosis. HSC were first described by Kupffer in the 19th century and their presence was confirmed by Rothe a decade later (Friedman, 2008a). The past thirty years have seen vast advances in the field of HSC biology and liver fibrosis. Most of the current knowledge of HSC is based on experiments done using animal models. Liver fibrosis can be induced in rodent models using either chemical mediated liver injury (e.g. CCl_4) or biliary fibrosis induced by bile duct ligation. Several animal cell lines have been set up to overcome the need to isolate HSC from animal models of liver fibrosis and facilitate *in vitro* assays (Gutierrez-Ruiz and Gomez-Quiroz, 2007). It is however, essential to validate the findings of animal studies in human HSC to determine their relevance in human disease. The limitation of using human HSC is the infrequent availability of 'healthy' human liver tissue, along with the unpredictable yield, purity and the variation in behavior of cells isolated from each donor. Immortalised human HSC lines have been used for the past decade to overcome these issues.

The most commonly used human HSC line is the LX2 cell line that has retained some key features of activated primary HSC (Xu et al., 2005). LX2

were created by Scott Friedman's group using primary human HSC from one donor and transfecting them with the pRSV-Tag plasmid that encodes the SV40 large T antigen under the control of rous sarcoma virus promoter. SV40 large T antigen has the capacity to inhibit G1 cell cycle growth arrest, rendering cells immortal (Ali and DeCaprio, 2001). These cells were then cultured in low serum media to further separate them from primary HSC that thrive on serum growth factors. Low (1%) and normal (10%) serum supplemented cultures were sequentially repeated to obtain the LX2 cell line, which has the ability to sustain itself and proliferate, even in low serum media, for up to fifty passages (Xu et al., 2005). LX2 have been shown to express typical markers of activated HSC such as αSMA, vimentin, PDGF-Rβ, ICAM-1 etc. In contrast to primary activated HSC, LX2 express GFAP and lack the expression of TIMP-1 (Xu et al., 2005).

As has been described in detail in Chapter 1, some cells of the immune system have been shown to play a pro-fibrogenic role whilst others have been attributed to have an anti-fibrotic function. Cells of the immune system that induce or perpetuate fibrosis include platelets, NKT cells, CD4 T cells, CD8 T cells, ILC, macrophages and DC. They do so by producing various pro-fibrogenic chemokines, cytokines and growth factors such as IL-13, IL-8, IL-4, IL-17, TNF-α, TGF-β and PDGF. In CHB, due to prolonged and persistent antigen exposure, the cells of the immune system have an altered phenotype and function as has been described by various groups in the past two decades. Whether immune cells in CHB have the capacity to produce soluble

mediators that contribute to CHB-associated liver fibrosis has never been studied before and is the main focus of this chapter.

Studies conducted by our group in HCV have demonstrated that peripheral mononuclear immune cells from patients with chronic hepatitis C produce soluble factors that activate HSC and their propensity to do so was associated with the rate of progression of fibrosis in these patients. Work done in the Rosenberg group by Fowell et al. demonstrated that PBMC from HCV infected patients with fast progressing fibrosis, on stimulation with HCV proteins (Core, NS3, NS4), are able to activate LX2 significantly more than those from HCV infected patients with slow progressing fibrosis (as shown in below in fig 3.1) or healthy controls (data not shown) (Fowell, 2008). I contributed to this work by performing RT PCR for markers of activation on LX2.

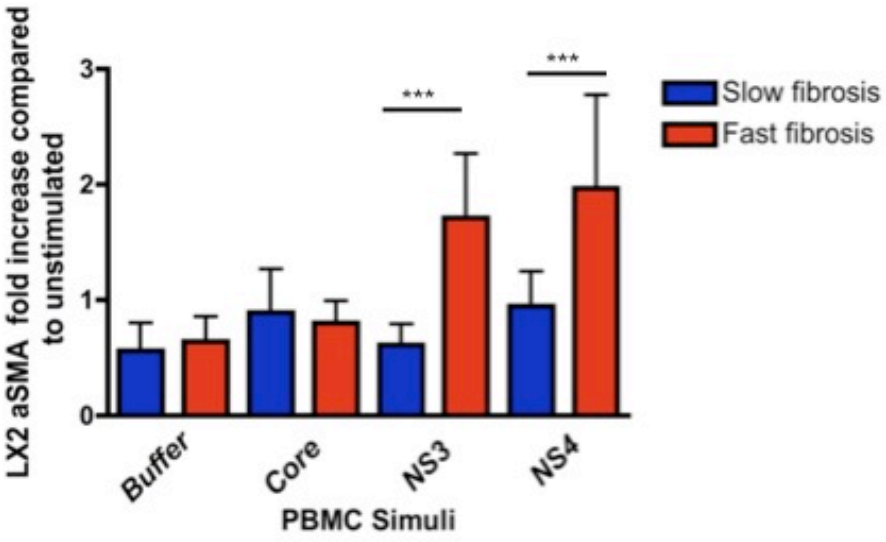


Figure 3-1: Effect of soluble mediators produced by PBMC from HCV infected patients on activation of LX2.

PBMC from HCV infected patients with fast and slow progressing fibrosis were stimulated with HCV proteins (Core, NS3 and NS4). The supernatant from the culture was collected 5 days post culture and applied to LX2 cells for 24 hours. The mRNA from LX2 was extracted and analysed for markers of activation using RT PCR. PBMC from HCV infected patients with fast progressing fibrosis were able to activate LX2 significantly more than from patients with slow progressing fibrosis. (p=0.0005, one-way ANOVA)

Lopes et al. from the Maini group performed gene array experiments using virus-specific CD8+ T cells from CHB patients and compared them with CD8+ T cells from patients who had resolved HBV (Lopes et al., 2008). They observed the upregulation of multiple pro-fibrogenic genes listed below (Table 3.1).

Gene Name	Unigene ID	Score(d)	Fold change
Connective tissue growth factor	75511	3.52	7.34
Extracellular matrix protein 2	35094	4.09	3.08
Fibroblast growth factor 2	284244	3.15	4.01
Profilin 1	75721	3.49	4.12
Procollagen (type III) N-endopeptidase	183138	3.70	2.69
Insulin-like growth factor binding protein	77326	4.23	9.25
Matrix metalloproteinase 17	159581	3.62	4.28

Table 3-1: Upregulation of fibrogeinc genes in virus-specific CD8+ T cells from CHB patients. Fold change and significance (d score) of genes in virus-specific CD8+ T cells from chronic HBV infected patients over resolved HBV infected patients.

These data from our group suggest that, as in chronic HCV infection, in CHB, some immune cells may exert a pro-fibrotic role. Results of the gene array study suggests that virus-specific CD8+ T cells in CHB may play a crucial role in fibrosis. This has never been studied in the context of CHB and was the main aim of this chapter.

3.1 Patient cohort

Local and national research ethics committees at Camden Primary Care Trust, University College Hospital, Royal London Hospital and Royal Free Hospital Trust approved this study. All participants of the study have given written, informed consent. All sample and data storage complies with the requirements of Data Protection Act 1998 and Human Tissue Act 2004. Peripheral blood was obtained from patients infected with CHB attending clinics at Mortimer Market Centre (Bloomsbury, London), University College London Hospital NHS Foundation Trust (Bloomsbury, London), Royal London Hospital, Barts Health NHS Trust (Whitechapel, London) and Royal Free London NHS Foundation Trust (Hampstead, London). All HBV infected patients were anti-HCV and anti-HIV antibody negative. Patients were stratified on the basis of their disease status: viral load (determined by real-time PCR), liver inflammation indicated by serum levels of alanine transaminase (ALT) and degree of fibrosis determined by Ishak stage and/or ELF test (Ishak score: 1-2 mild fibrosis, 3-4 moderate fibrosis, 5-6 cirrhosis; ELF test: ≤7.7 no/mild fibrosis, 7.7-9.8 moderate to severe fibrosis, >9.8 cirrhosis).

Table 3.2:

Patient ID	HBeAg status	Fibrosis status	Viral load (IU/ml)	ALT (IU/L)	Gender	Age (years)	Treatment
Pt01	+	Moderate (Biopsy)	700,000,000	66	M	71	Untreated
Pt02	+	Mild (biopsy)	80,275.14	36	F	33	Untreated
Pt03	+	Normal (ultrasound)	51,406,000	330	F	23	Untreated
Pt04	-	Mild (Biopsy)	31,344	37	F	25	Untreated
Pt05	-	Not done	720	41	M	26	Untreated
Pt06	+	Moderate (Biopsy)	100,000,000	87	F	32	Untreated
Pt07	-	Mild (ELF)	849	43	M	39	Untreated
Pt08	-	Mild (ELF)	31	15	F	34	Untreated
Pt09	-	Not done	137	11	F	49	Untreated
Pt10	-	Moderate (ELF)	66	15	F	54	Untreated
Pt11	+	Mild (ELF)	7,022,700	108	F	27	Untreated
Pt12	-	Moderate (Biopsy)	48,021	54	F	68	Untreated
Pt13	-	Mild (ELF)	80	31	F	22	Untreated
Pt14	-	Moderate (Biopsy)	6583	27	F	41	Untreated
Pt15	-	Cirrhotic (Biopsy)	Not detected	200	M	70	Untreated
Pt16	+	Cirrhotic (Biopsy)	1,990,429	44	F	62	Untreated

Pt17	-	Mild (Biopsy)	828,773	281	F	37	Untreated
Pt18	+	Mild (Biopsy)	1,545,586	37	Not known	49	Untreated
Pt19	-	Mild (Biopsy)	421	58	M	46	Untreated
Pt20	Not done	Moderate (ELF)	48	48	M	37	Untreated
Pt21	+	Moderate (ELF)	104,891	22	F	25	Untreated
Pt22	-	None (Biopsy)	1092	53	F	37	Untreated
Pt23	-	Normal (Biopsy)	41,728	95	M	41	Untreated
Pt24		Normal (fibroscan 3.6)	11,000	33	M	41	Untreated
Pt25	-	Not done	1119	29	M	45	Untreated
Pt26	-	Not done	4,400	59	M	46	Untreated
Pt27	-	Not done	Not detected	17	F	61	Untreated
Pt28	-	Moderate (Biopsy)	1150	114	Not known	49	Untreated
Pt29	-	Moderate (Biopsy)	142,919	48	Not known	32	Untreated
Pt30	-	Not done	Not detected	11	F	35	Untreated
Pt31	+	Mild (Biopsy)	249,497,143	38	F	30	Untreated
Pt32	+	Mild (Biopsy)	1,115,000,000	78	M	30	Untreated
Pt33	-	Moderate (Biopsy)	24	26	M	37	Untreated
Pt34	-	Normal	7678	68	M	50	Untreated

		(Biopsy)					
Pt35	-	Not done	Not detected	normal	Not known		Untreated
Pt36	-	Not done	Not detected	normal	M	50	Untreated
Pt37	+	Not done	2,000,000	230	F	42	Untreated

Table 3-2: CHB patient cohort detail information.

Details of CHB patients used in the study. First column is the patient study code, second column shows the HBeAg status of the patient, third column denotes the fibrosis stage of the patient, fourth column is the HBV DNA (viral load) of the patients (IU/ml), fifth column gives alanine aminotransferase (ALT) levels of the patient in IU/L, sixth column indicates the gender of the patient (Female=F, Male=M), seventh column indicates the age of the patient at the time of sample collection in year and the eighth column indicates the treatment status of the patient at the time of sample collection,

The details of normal healthy donors that consented and participated in this study are as follows.

Table 3.3:

Donor ID	Gender	Age
HD01	M	37
HD02	F	31
HD03	F	25
HD04	F	38
HD05	F	39
HD06	F	26
HD07	M	21
HD08	M	38
HD09	F	31
HD10	M	30

Table 3-3: Healthy donor detailed information.

The first column gives the study code of the healthy donors, second column gives details of gender of the participant and the third column gives details of the age at the time of sampling of each participant.

Based on the background data described, we set out to investigate whether PBMC from patients with CHB can produce soluble factors driving a pro-fibrogenic reaction in stellate cells. To this end, we performed experiments using a transwell system. We cultured PBMC from CHB patients for 9 days, with and without stimulation, with HBV peptides before transferring to the upper well of transwell chambers, and LX2 were cultured in the lower chamber twenty four hours prior. Twenty four hours after culture in the transwell system with PBMC in the upper chamber, the activation status of LX2 cells in the lower chamber was examined at protein level (CCL2, ICAM1) using flow cytometry. In addition, the PBMC supernatants were collected and applied to a fresh batch of LX2 for 16hrs and the levels of activation markers (αSMA and Pro-collagen I) of the LX2, relative to un-stimulated LX2, were measured at an mRNA level using real-time PCR. GAPDH was the house-keeping gene used for RT PCR assays.

3.2 Activation of stellate cells at mRNA levels by soluble mediators produced by PBMC of CHB patient

As mentioned above, PBMC from CHB patients and healthy volunteers were cultured with LX2 in a transwell system for 24 hours. The supernatant from this system was collected and applied to a fresh batch of LX2, cultured in 48-well tissue culture plates at a density of 5,000 cells/cm^2 for 16 hours. The LX2 were then harvested and their RNA extracted and converted to cDNA. The levels of αSMA and Pro-collagen I were then determined using Real-time PCR as has been described in further detail in Materials and Methods section of this thesis. Figure 3.2a illustrates the experimental setup. We found a statistically significant increase in the mRNA levels of αSMA in LX2 that were cultured in supernatants from CHB patient PBMC compared to healthy PBMC (Fig 3.2b). We found a similar trend in Pro-collagen I mRNA levels (Fig 3.2c), however this was not statistically significant. This suggests that PBMC from CHB patients are in a pro-fibrogenic state and are able to produce soluble mediators that can activate stellate cells compared to PBMC from healthy controls.

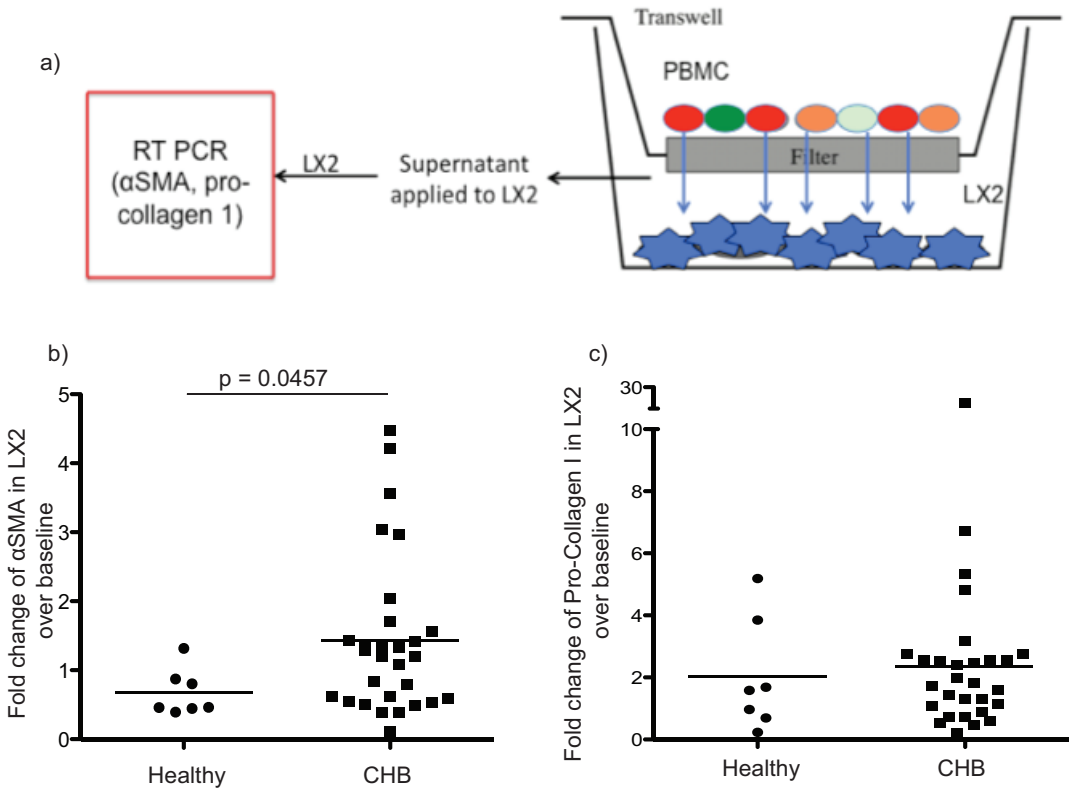


Figure 3-2: Activation of stellate cells at mRNA levels by soluble mediators produced by PBMC of CHB patients.

a) Diagram of experimental setup. PBMC from CHB patients and healthy donors were cultured for 9 days and transferred to the top chamber of the transwell system, LX2 were grown in the bottom panel of the transwell system for 24 hours. The supernatant was collected and applied to fresh LX2 grown in a separate plate for 16 hours. These LX2 were then harvested and their mRNA was extracted using Taqman Cell-to-CT kit. Real time PCR was performed to look for the mRNA expression of αSMA and Pro-Collagen I. b) Cumulative data showing the fold change in mRNA levels of αSMA in LX2 cultured with supernatants from PBMC of healthy donors and CHB patients over baseline levels normalised to GAPDH. Significantly higher up-regulation of αSMA in LX2, cultured with PBMC from CHB patients compared to healthy donors (p=0.0457, Mann-Whitney U-test) c) Cumulative data showing the fold change in mRNA levels of Pro-Collagen I in LX2 cultured with supernatants

from PBMC of healthy donors and CHB patients over baseline levels normalized to GAPDH (p=0.6033, Mann-Whitney U-test).

3.3 Clinical parameters of CHB patients do not influence their capacity to produce fibrogenic soluble mediators that activate stellate cells at mRNA level

We found a difference between activation of LX2 subjected to supernatants from CHB patients compared to healthy controls, however, there was a spread observed in the potential of PBMC from CHB patients to activate HSC. To understand this better, CHB patients were stratified on the basis of clinical parameters. The CHB patients were divided on the basis of their fibrosis stage (Ishak score cut off 2, ELF test cut off 7.7), viral load (cut off 2,000 IU/ml) and the levels of ALT (cut off 50 IU/L). There was no significant difference observed in the levels of αSMA in patients with varying levels of fibrosis, viral load or ALT (Figure 3.3a).

The same analysis was performed for Pro-collagen I and no significant differences were found in any of the clinical parameters (Figure 3.3b).

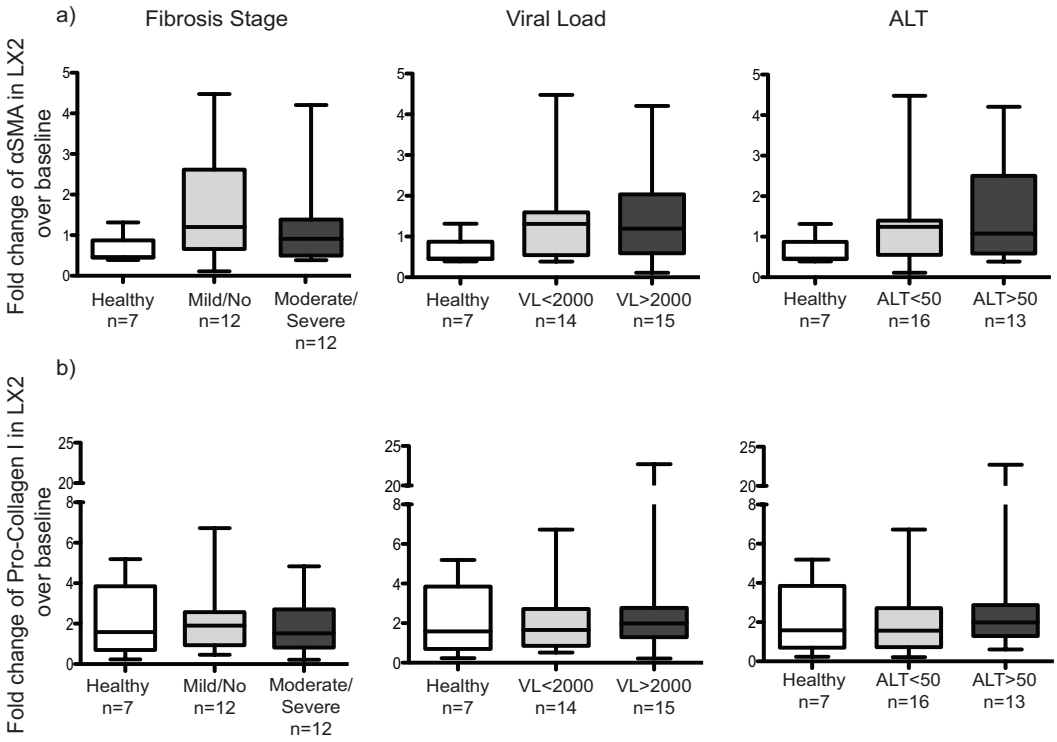


Figure 3-3: Clinical parameters of CHB patients do not reflect on their capacity to produce soluble mediators to activate LX2.

RT PCR performed on LX2 incubated with supernatants from PBMC cultures of healthy donors and CHB patients to examine the levels of αSMA and Pro-collagen I. CHB patients are divided on the basis of their fibrosis stage, viral load (VL) and ALT levels. a) no statistical difference was found when CHB patients were divided on the basis of fibrosis stage (p=0.1509, Kruskal Wallis ANOVA), viral load (p=0.1269, Kruskal Wallis ANOVA) and ALT levels (p=0.1266, Kruskal Wallis ANOVA) and their potential to upregulate αSMA at mRNA levels was examined. b) no statistical difference found when CHB patients were divided on the basis of fibrosis stage (p=0.9006, Kruskal Wallis ANOVA), viral load (p=0.8172, Kruskal Wallis ANOVA) and ALT levels (p=0.4830, Kruskal Wallis ANOVA) and their potential to upregulate Pro-collagen I at mRNA levels was examined.

3.4 Optimisation of the characterisation of HSC using flow cytometry

Typically, as a read out in studies conducted using LX2 and/or primary HSC (animal or human), markers of activation are either examined at mRNA level using RT PCR or at protein level using western blots or microscopy associated techniques such as immunofluorescence, immunohistochemistry and immunocytochemistry. These are all very widely used, validated techniques however they are unable to provide a qualitative and quantitative assessment of the expression of various markers on each cell. To address this we developed the characterisation of LX2 using flow cytometry (FACS). It is worth noting that stellate cells are comparatively large in size, along with being auto-fluorescent when quiescent, making them difficult to study using FACS. For this reason, LX2 were cultured and stained for markers of activation to determine if FACS can be used as an assay to quantify HSC activation. The markers that were chosen were αSMA, a marker expressed by HSC when they become myofibroblastic; ICAM1, an adhesion molecule expressed on activated HSC; CCL2, also known as monocyte chemo-attractant protein-1 (MCP-1), a chemokine produced by HSC to facilitate the migration lymphocytes to the site of injury; and PDGFβ receptor, up-regulated on activated HSC to promote their proliferation. Plots in Figure 3.4 demonstrate baseline levels of activation of LX2 determined by the expression of these markers compared to their respective antibody isotypes using FACS. The plots show the frequency of expression of these markers at baseline and their mean fluorescence intensity against isotype controls.

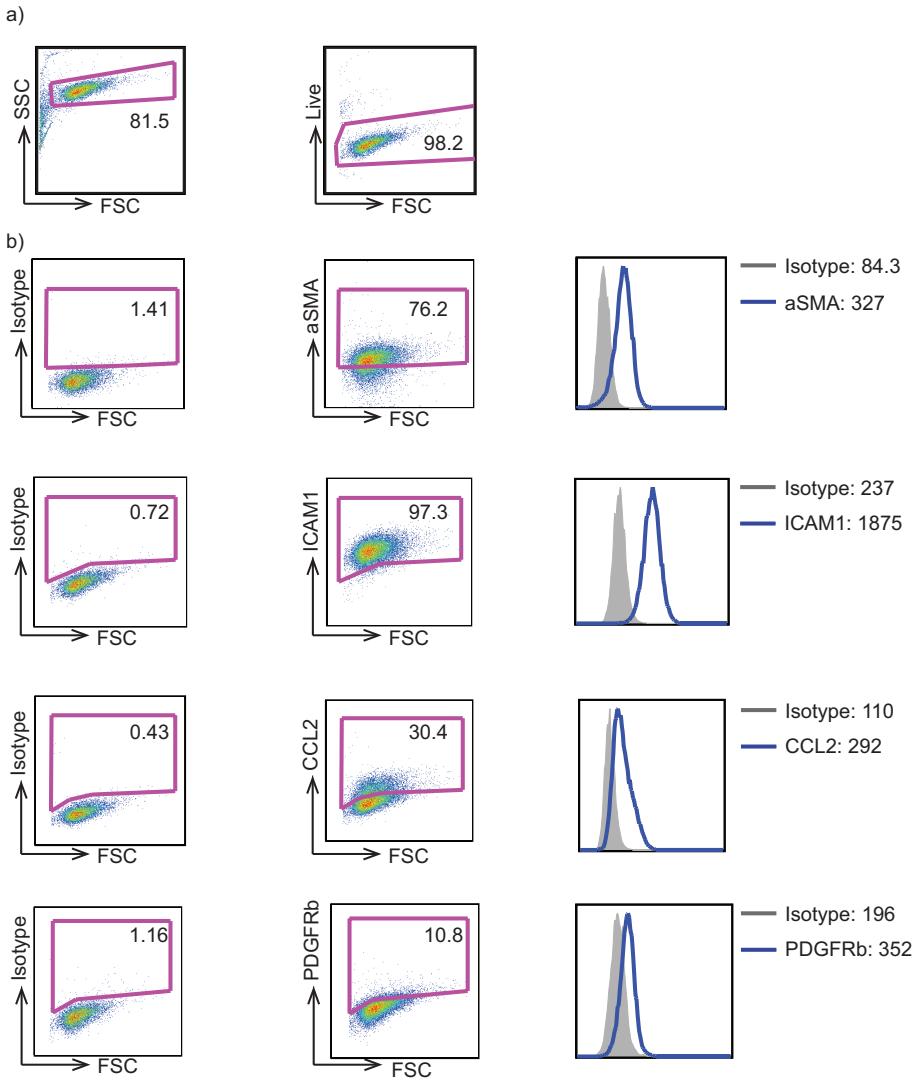


Figure 3-4: Characterisation of LX2 cells using FACS.

LX2 were stained for Dead cell marker and markers of activation: αSMA, ICAM1, CCL2 and PDGFRβ along with respective isotypes. The cells were acquired using flow cytometry on BD LSR2 and analysed using Flowjo software. a) Gating strategy of HSC showing forward scatter/side scatter (FSC/SSC) gates and Live/Dead gates. b) Representative plots and histograms showing percentage expression and MFI respectively of activation markers on HSC against matched isotype controls.

3.5 Activation of stellate cells at protein level by soluble mediators produced by PBMC from CHB patient

To investigate the activation of stellate cells at protein level, multi-colour flow cytometry was used as a read out. αSMA, PDGFRβ, CCL2 and ICAM1 were stained for, as markers of activation as has been described in the previous sub-section. However, it was found that the staining for αSMA and PDGFRβ was inconsistent between experiments and we are trying to optimise this. For this reason, these data were excluded from current data analysis. In the following data, levels of chemo-attractant CCL2 and adhesion molecule ICAM1 were studied as markers of activation.

To investigate the potential of PBMC from CHB patients and healthy donors to produce soluble mediators that can activate HSC at protein level, PBMC from CHB patients and healthy donors were cultured for 9 days. They were then transferred to the top chamber (permeable membrane) of the transwell system. The LX2 were plated on the bottom chamber of the transwell system 24 hours prior to culture with PBMC. This transwell culture was incubated for 24 hours and LX2 were harvested to stain for markers of activation (ICAM1 and CCL2) and were acquired for flow cytometry using the BD LSR2. The data was analysed using FlowJo software. A diagram of the experimental design is illustrated in figure 3.5.

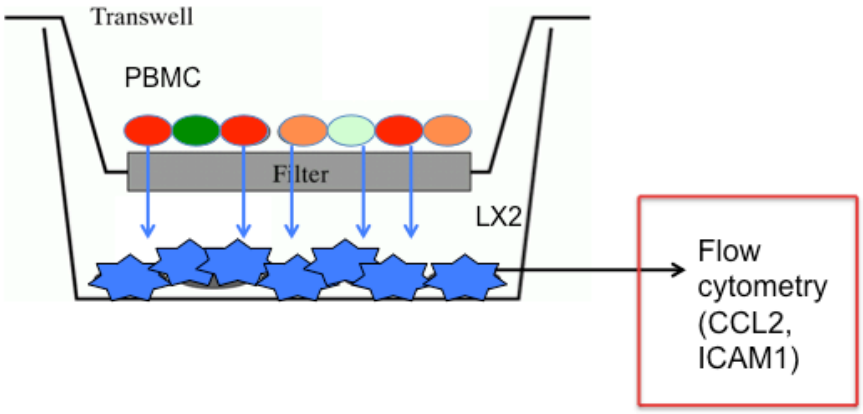


Figure 3-5: Experimental design.

LX2 were plated at the bottom and PBMC were plated in the top chamber of the Transwell system for 24 hours. At the end of incubation, LX2 were harvested using Trypsin/EDTA and were stained for markers of activation (ICAM1 and CCL2) and analysed using flow cytometry.

As seen in the representative plot in Figure 3.6a, the levels of CCL2 and ICAM1 on LX2 were increased more than two-fold, when they were cultured with PBMC from CHB patients compared to LX2 alone as opposed to minimal/no difference between LX2 cultured with healthy PBMC versus LX2 alone. In the cumulative data shown in Figure 3.6b, there was a trend towards increased levels of CCL2 and ICAM1 in LX2, cultured in a transwell system with PBMC, from CHB patients compared to PBMC from healthy, however this was not statistically significant. This is similar to the trends observed at mRNA level (Figure 3.2) when the levels of αSMA and Pro-collagen I were examined, suggesting that PBMC from CHB patients produce soluble mediators that have a variable capacity to activate hepatic stellate cells at mRNA and protein levels.

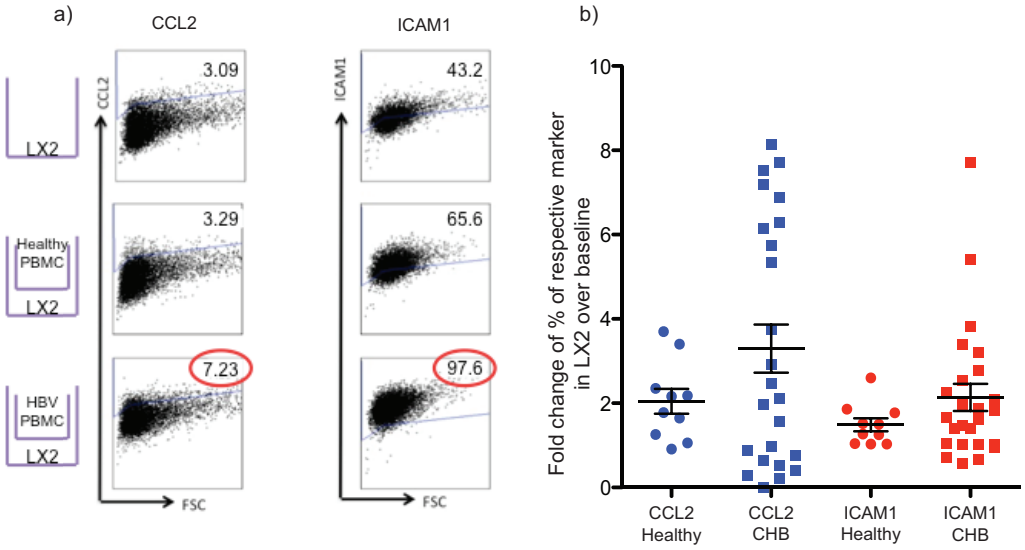


Figure 3-6: PBMC from CHB patients have a variable capacity to activate HSC by producing soluble mediators.

Fold change over baseline of activation of LX2 upon culture with PBMC from healthy donors and PBMC from CHB patients in a transwell system. LX2 were stained for respective markers and flow cytometry was used analyse data. a) No statistical difference was found when CHB patients were divided on the basis of fibrosis stage (p=0.3507, Kruskal Wallis ANOVA), viral load (p=0.9192, Kruskal Wallis ANOVA) and ALT levels (p=0.4323, Kruskal Wallis ANOVA) and their potential to upregulate CCL2 at protein levels was examined. b) no statistical difference found when CHB patients were divided on the basis of fibrosis stage (p=0.6174, Kruskal Wallis ANOVA), viral load (p=0.6661, Kruskal Wallis ANOVA) and ALT levels (p=0.7235, Kruskal Wallis ANOVA) and their potential to upregulate ICAM1 at protein levels was observed.

3.6 Clinical stratification of CHB patients do not influence their capacity to produce fibrogenic soluble mediators that activate stellate cells at protein level

CHB patients were then segregated on the basis of clinical parameters. They were divided on the basis of their fibrosis stage (Ishak score cut off 2, ELF test cut off 7.7), viral load (cut off 2000 IU/ml) and the levels of ALT (cut off 50 IU/L). Even though a trend towards the increased levels of CCL2 and ICAM1 in LX2, cultured in a transwell system with PBMC from CHB patients compared to healthy was observed; no statistical difference was found in the levels of activation marker in LX2 between CHB patients with varying degree of fibrosis, viral load and ALT (Figure 3.7). This was consistent with the trends observed when levels of activation at mRNA level were studied after stratifying the CHB patients on the basis of their clinical parameters (Figure 3.3).

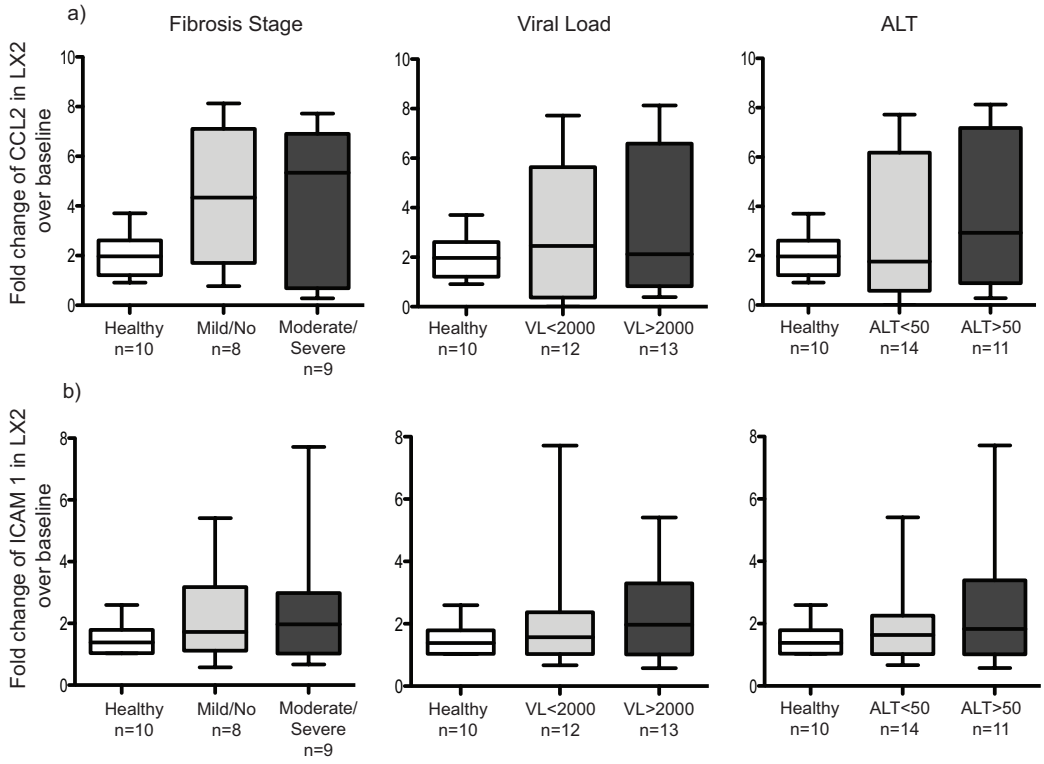


Figure 3-7: Clinical parameters of CHB patients do not reflect on their capacity to produce soluble mediators to activate LX2 at protein level.

Fold change over baseline of activation of LX2 upon culture with PBMC from healthy donors and CHB patient PBMC, in a transwell system. LX2 were stained for respective markers and flow cytometry was used for analysis. a) No statistical difference was found when CHB patient were divided on the basis of fibrosis stage (p=0.3507, Kruskal Wallis ANOVA), viral load (p=0.9192, Kruskal Wallis ANOVA) and ALT levels (p=0.4323, Kruskal Wallis ANOVA) and their potential to upregulate CCL2 at protein levels was examined. b) no statistical difference was found when CHB patients were divided on the basis of fibrosis stage (p=0.6174, Kruskal Wallis ANOVA), viral load (p=0.6661, Kruskal Wallis ANOVA) and ALT levels (p=0.7235, Kruskal Wallis ANOVA) and their potential to upregulate ICAM1 at protein levels was observed.

3.7 Investigating potential soluble mediators that may be produced by CHB patient PBMC that activate HSC

As has been described earlier, gene array studies done by our group have demonstrated that virus-specific CD8+ T cells from CHB patients upregulate certain pro-fibrogenic genes compared to CD8+ T cells from patients with resolved HBV. Table 3.1 enlists these genes along with their fold change increase. Connective tissue growth factor (CTGF) and insulin-like growth factor binding protein 3 (IGFBP3) had the highest fold change in gene expression on virus specific CD8+ T cells from CHB patients compared to patients with resolution of HBV.

CTGF is a monomeric protein that comprises 349 residues (Bradham et al., 1991). It has been shown to be upregulated by TGF-β, hypoxia and high levels of glucose (de Winter et al., 2008; Gressner et al., 2007; Phanish et al., 2005). CTGF has been shown to be found in cytosolic space in mesangial cells, however mostly CTGF is secreted and can be found in extracellular spaces and the micro-environment of cells. It can be detected in plasma, serum and urine (Gressner and Gressner, 2008; Kubota et al., 2004; Roestenberg et al., 2006). Several studies have implicated the role of CTGF in cell proliferation, migration, matrix production and cellular adhesion in various cells in the body (de Winter et al., 2008; Safadi et al., 2003; Secker and Daniels, 2008; Twigg et al., 2002; Weston et al., 2003).

Insulin-like growth factors (IGF) have been reported to play a role in cell growth, differentiation and metabolism. In the circulation, they are usually bound to Insulin-like growth factor binding proteins (IGFBP) that facilitate the attachment of IGF to their receptors and also increase their half life (Ruan and Ying, 2010). High expression of IGFBP3 has been associated with pulmonary fibrosis and cystic fibrosis (Canale-Zambrano and Haston, 2011; Pilewski et al., 2005). Their role in liver fibrosis remains to be investigated.

While performing experiments using PBMC from CHB patients and culturing them with LX2 in a transwell system, supernatant from some of the assays were frozen. The supernatants were thawed and ELISA performed to determine the levels of CTGF and IGFBP3 in the supernatants. Commercially bought ELISA kits were used. As can be seen in Figure 3.8 below, the PBMC from CHB patients produce CTGF in general. In this cohort the levels of CTGF produced are not influenced by the disease status of the patient (Figure 3.9). It is noteworthy that the cohort is small and this experimental assay needs to be performed on a larger cohort to have a better understanding of the role of CTGF production by PBMC of CHB patients, and to examine if their clinical parameters influence their production of CTGF. ELISA was also performed for IGFBP3, however the optical densities for all the levels were below levels of detection (data not shown) suggesting that this protein is not secreted by PBMC from CHB patients in culture. Alternatively, ELISA as an assay is not sensitive enough to detect the levels of any secreted IGFBP3.

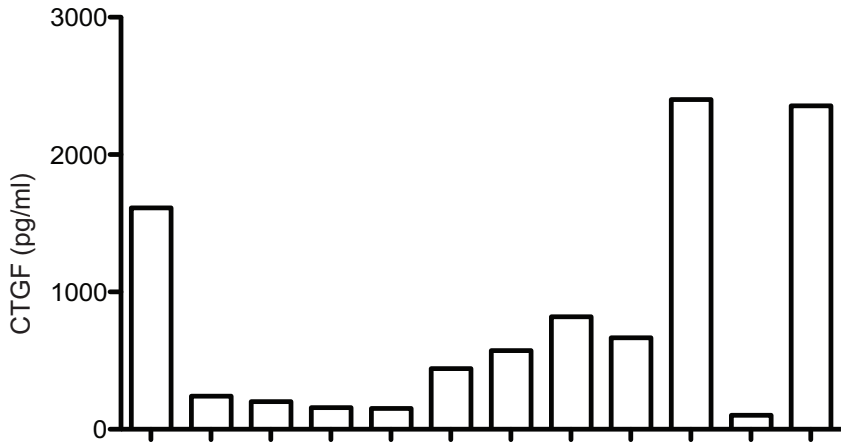


Figure 3-8: Levels of CTGF produced by PBMC from CHB patients produced in culture.

ELISA was performed on supernatant collected from PBMC cultured with LX2 in a transwell system using a commercially available kit following manufacturers instructions. This graph shows levels of CTGF (pg/ml) detected in the supernatant of PBMC culture of each patient. Each individual bar represents one patient.

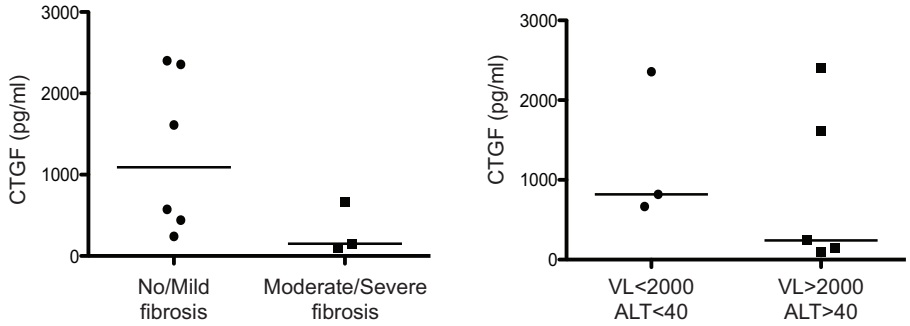


Figure 3-9: Clinical parameters of CHB patients do not correlate with their capacity to produce CTGF.

CHB patients from figure 2.10 were segregated on the basis of their fibrosis stage, VL and ALT as described previously. No statistically significant

difference was found when CHB were compared on the basis of their fibrosis stage (p=0.1667, Mann Whitney U-test) or on the basis of their viral load (VL) and ALT (p=0.5714, Mann Whitney U-test).

3.8 Activation of HSC by PBMC from CHB patients is not mediated by HBV peptides

To determine whether the capacity of PBMC from CHB patients to activate HSC was antigen specific, PBMC from CHB patients were stimulated with immunodominant HBV overlapping peptides (pool of 15mer peptides overlapping by 10 residues spanning 24 peptides of the core of HBV) on day zero of culture and were re-stimulated with the peptides on day 9, when they were added to the top chamber, of the transwell system.

It was observed that PBMC from CHB patients on stimulation with HBV overlapping peptides did not have an enhanced effect on the activation of LX2 in a transwell culture system at mRNA level as shown in Figure 3.10.

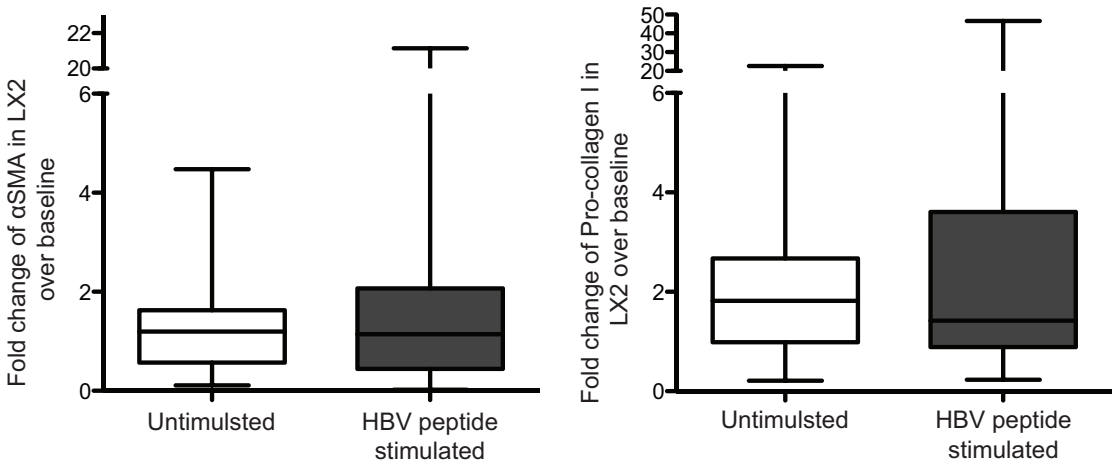


Figure 3-10: HBV peptide stimulation of PBMC from HBV infected patients has no effect on their fibrotic potential at mRNA level.

PBMC from CHB patients (n=29) did not augment their pro-fibrotic effect on stimulation with HBV overlapping peptides as there is no difference seen in the mRNA levels of αSMA (p=0.7621, Wilcoxon paired test) and the mRNA levels of pro-collagen I (p=0.8797, Wilcoxon paired test) activation of LX2. The mRNA levels are calculated as a fold change of baseline normalized to GAPDH.

Next we wanted to examine if this lack of further activation of LX2, after transwell system culture, with PBMC from CHB patients stimulated with HBV overlapping peptides was also translated to protein level. For this PBMC from CHB patients were stimulated with immunodominant HBV overlapping peptides on day zero of culture and were re-stimulated with the peptides on day 9, when they were added to the top chamber of the transwell system. The LX2 plated at the bottom chamber of the transwell system were harvested 24 hours later and were stained for CCL2 and ICAM1 as markers of activation. The data were analysed using flow cytometry.

It was observed that PBMC from CHB patients on stimulation with HBV overlapping peptides did not have an enhanced effect on the activation of LX2 in a transwell culture system at protein level as well as shown in Figure 3.11.

The HBV overlapping peptides are able to stimulate both CD4+ and CD8+ T cell responses. This suggests that a non virus-specific cell type is producing the fibrogenic soluble mediators. An alternative interpretation is that the response elicited by this strategy in these patients was insufficient to stimulate HSC above what is seen without in vitro re-stimulation. These possibilities are discussed further in the conclusion of the chapter.

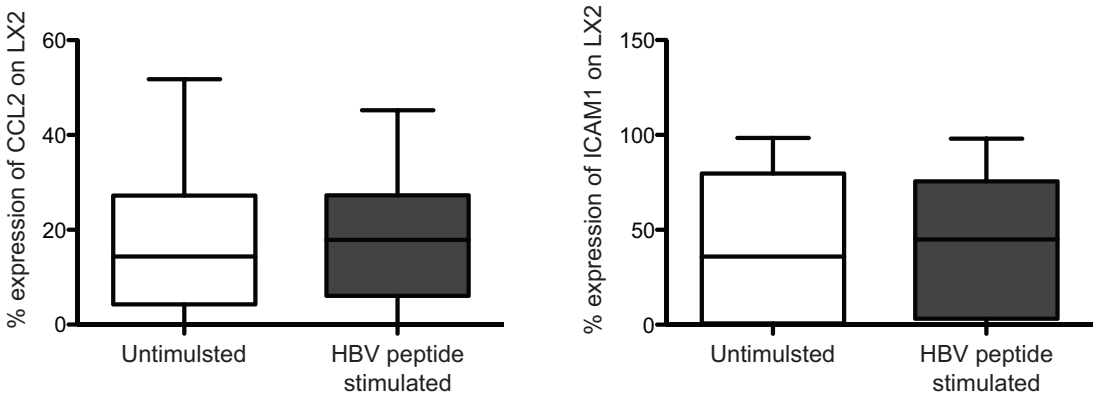


Figure 3-11: HBV peptide stimulation of PBMC from HBV infected patients has no effect on their fibrotic potential at protein level.

PBMC from CHB patients (n=25) did not augment their pro-fibrotic effect on stimulation with HBV overlapping peptides as there is no difference seen in the protein levels of CCL2 (p=0.5360, Wilcoxon paired test) and protein levels of ICAM1 (p=0.0.1194, Wilcoxon paired test) as markers of activation of LX2. The CCL2 and ICAM1 levels are shown as percentage expression of each respective marker.

Conclusion

In this chapter the potential of PBMC from CHB patients to produce soluble mediators that may activate HSC was investigated. This was done using PBMC from CHB patients and healthy controls and culturing them in a transwell system with a well-established human HSC cell from Scott Friedman's group called the LX2. The readouts were both at mRNA levels and protein level.

LX2 is an activated HSC line, however, as has been previously described, bu our group and others, that LX2 can be further activated.

It was observed that PBMC from CHB patients can significantly upregulate mRNA levels of αSMA and show a trend towards the upregulation of pro-collagen I. As a spread was observed in the potential of LX2 to be activated by PBMC from CHB patients, the CHB were then segregated on the basis of their fibrosis stage, viral load and ALT levels. No significant differences were detected.

This effect was also studied at protein level and cumulatively a trend was observed in the potential of PBMC of CHB patients to activate HSC, however this was not statistically significant. In line with observations made at mRNA level, dividing patients on the basis of clinical parameters did not show any significant differences in their potential to activate HSC.

This could reflect that the clinical parameters used in this study to stratify the patients for analysis do not correlate with their potential to activate HSC. It would be interesting to classify patients on their disease stage as has been described by the European Association of Study of the Liver (EASL) (described in detail in chapter 1). However, for this clinical data from the patients would have to be collected at sequential time points to determine their disease progression. These clinical data are not available to us at this point.

To further investigate previous published data from the group by Lopes et al. in the context of this project, we wanted to try and identify some potential soluble mediators produced by PBMC of CHB patients that could be activating HSC. CTGF and IGFBP3 were selected to be investigated, as the genes of these two proteins were highly upregulated on virus-specific CD8+ T cells. ELISA were performed on the supernatants of PBMC cultures for CTGF and IGFBP3 (Lopes et al., 2008). CTGF was found to be produced by PBMC of all donors and this was not dependent on their CHB clinical stratification. IGFBP3 was not detected in the supernatant of healthy donors or patient PBMC. This suggests that some other soluble mediators may play a role in activating HSC.

To determine whether this activation was an effect of virus-specific cells, the PBMC from CHB patients were stimulated with immunodominant HBV overlapping peptide that stimulate both CD4+ and CD8+ T cell responses.

However, no differences were found in the HSC activation capacity of unstimulated and peptide stimulated PBMC. These data imply that a non-virus specific cell type is producing the soluble mediators that are further activating LX2.

The findings of this chapter are discussed in further detail in the discussion chapter.

4. Assessing the potential of NK cells from patients with CHB to kill hepatic stellate cells

Background

As has been described in the previous chapter, most of the published human *in vitro* studies using HSC have been conducted using a human HSC cell line – LX2. This is due in part to lack of availability of human liver tissue to extract primary human HSC and also because the a standardized cell line provides a constant and reproducible tissue for research. Fortunately, during the course of this study, this lab had the opportunity of access liver tissue from healthy margins of liver tissue following resections for hepatic metastases. These healthy margins have been used to optimise the isolation of primary HSC. Making full use of this rare opportunity, the major experiments of the next two chapters were done using primary human HSC. LX2 were used to optimise, compare and characterize assays. Being one of the few labs in the world to have access to human healthy liver tissue and use primary human HSC, the various assays used in this these chapters had to be carefully optimised and corrected to account for inter-donor variability. The details of this have been discussed in the next section.

The crucial role of the HSC in the liver injury response and fibrogenesis has been discussed in greater detail in the Introduction section. In acute liver injury HSC become activated and accumulate at the site of injury (Iredale et

al., 1998; Mathew et al., 1994). Upon activation, HSC acquire a more myofibroblast-like αSMA-positive phenotype and start to proliferate, which has been attributed to being central to the entire fibrotic process (Pinzani and Rombouts, 2004). Activated HSC express collagen I and III (Friedman, 2008b; Friedman et al., 1985). HSC have been shown to also express MMPs such as MMP-1, MMP-2 and MMP-14 that can degrade collagenous matrix (Aimes and Quigley, 1995; Milani et al., 1994; Ohuchi et al., 1997). It has been demonstrated in animal models and human studies that progression of fibrosis is associated with reduced interstitial collagenase activity of MMPs and this has been attributed to the production of TIMP-1 and TIMP-2 by HSC (Arthur, 2000; Iredale, 2001). When activated, HSC enter the growth cycle (Friedman, 2008a) which leads to an overall increase in the number of activated HSC that actively produce collagenous matrix and simultaneously prevent its degradation by expressing TIMPs, as is seen in chronic livery injury. Resolution of fibrosis has been observed in both acute and chronic fibrosis once the underlying condition/disease is successfully cleared or treated (Dufour et al., 1997; Dufour et al., 1998; Hammel et al., 2001; Mathew et al., 1994; Vukobrat-Bijedic et al., 2014). Resolution is associated with reduction of ECM and restoration of the liver architecture to a normal to near-normal state. For this, reduction in the number of activated HSC is essential. This is possible by three ways: reversion to a quiescence-like state, direct apoptosis and senescence-led apoptosis (Bataller and Brenner, 2005). Of these, apoptosis of activated HSC is the most widely researched and has been demonstrated to be the most effective and plausible mechanism of resolving fibrosis. Studies looking at the role of other cells contributing to their

apoptosis have revealed that, of the resident liver cells, KC stimulated with LPS can induce apoptosis of HSC in a caspase 9-mediated manner *in vitro* (Fischer et al., 2002). In addition, the crucial HSC apoptosis-triggering function of NK cells has come to light in several studies in the past decade (Gur et al., 2012; Radaeva et al., 2006).

NK cells belong to the innate arm of the immune system. They play a crucial role in acute infection, forming an initial defense against the pathogen. In chronic infections, they can have dysregulated effector function. NK cells can be activated on stimulation with cytokines such as type I IFNs, IL-12, IL-18, IL-15 and IL-8 and a downregulation of inhibitory signals (Nguyen et al., 2002; Rehermann, 2013). However, in CHB they are not just responsible for antiviral activity but have an important regulatory function throughout the chronic phase of disease (Rehermann, 2013). NK cells are enriched in the liver at the site of HBV viral replication (Doherty et al., 1999; Dunn et al., 2007). In CHB, NK cells can kill viral infected hepatocytes and contribute to viral clearance while causing liver damage (Dunn et al., 2007). They have also been shown to contribute to viral persistence by deleting virus-specific CD8+ T cells in CHB via the TRAIL pathway (Peppa et al., 2013). These studies demonstrate varying roles of NK cells in CHB.

NK cells have higher expression of activatory receptors NKp46, NKp30 and NKG2C in CHB compared to chronic HCV infected patients (Nattermann et al., 2006). They also have elevated expression of the death ligand TRAIL especially in patients with high ALT compared to healthy controls (Dunn et al.,

2009; Peppa et al., 2013). TRAIL is preferentially expressed on the $CD56^{bright}$ subset of NK cells that mediate non-antigen specific death via this pathway. The percentage of $CD56^{bright}$ NK cells is higher in the intrahepatic compartment compared to the periphery (Bonorino et al., 2009). During flares of CHB, it has been demonstrated that the IFN-α induced in this phase drives the expression of TRAIL on NK cells (Dunn et al., 2007). It has also been observed that CHB patients on pegylated IFN-α therapy have an increased expression of TRAIL on their NK cells (Micco et al., 2013).

Death ligand TRAIL has the capacity to bind to four membrane bound receptors: TRAIL R1 (DR4), TRAIL R2 (DR5, Killer, TRICK), TRAIL R3 (DcR1, LIT, TRID) and TRAIL R4 (DcR2, TRUNDD) and one soluble receptor, osteoprotegrin (OPG) (Lalaoui et al., 2011). TRAIL can induce apoptosis by binding to TRAIL-R1 and TRAIL-R2 on target cells. TRAIL-R1 and 2 both have an intracellular death domain (DD), which is essential for downstream signaling leading to apoptosis (Feinstein et al., 1995).

On binding with trimeric TRAIL, TRAIL-R1 and R2 induce the recruitment of FADD (Fas-associated death domain protein) with their respective DD, which leads to the recruitment of pro-caspase-8 and -10, resulting in the formation of DISC (death-inducing signaling complex). Within the DISC, caspase-8 and -10 undergo downstream catalytic cleavage in the cytosol triggering the caspase cascade which leads to caspase-3 mediated apoptosis (Bodmer et al., 2000; Kischkel et al., 1995; Kischkel et al., 2001).

The anti-fibrotic role of NK cells has been documented in several animal models over the past decade. Work done by the Friedman group, was the first to demonstrate that HSC can undergo TRAIL-mediated death using recombinant TRAIL (Taimr et al., 2003). In 2006, two important independent murine studies were the first to demonstrate the role of NK cells in HSC killing, and this was attributed to the TRAIL and the NKG2D pathway (Melhem et al., 2006; Radaeva et al., 2006). In the past few years, studies have moved on to primary human HSC and the Natterman group have demonstrated in *in vitro* cultures, the anti-fibrotic role of NK cells of HCV patients through TRAIL, NKG2D and Fas dependent killing of HSC (Glassner et al., 2013; Glassner et al., 2012). Collaborative work between the Safadi and Mandelboim groups has also shown that NK cells can kill HSC via the NKp46 pathway; they were the first to demonstrate expression of the unknown ligand of NKp46 on HSC using fusion proteins (Gur et al., 2012). However, the anti-fibrotic role of NK cells has never been investigated in the context of CHB. Keeping in mind the altered phenotype of NK cells in CHB, the main aim of this chapter is to investigate if NK cells from CHB have the ability to kill primary human HSC.

4.1 Optimising extraction and culture of primary human HSC

For the majority of the assays in this chapter and the next chapter, primary human HSC have been used. As has been mentioned previously, many of the studies related to human HSC are conducted using LX2 cell line. In our group, we had access to healthy margins of metastatic liver resections. These

resections were used to isolate primary human HSC that were expanded and stored for subsequent use in experiments. A detailed methodology of the extraction process has been described previously in the Materials and Methods section. Briefly, the liver resection is mechanically cut into small pieces (approximately 2mm) and digested using 0.1% collagenase type IV and 0.001% DNase I for 20 minutes. The homogenate is then passed through 70 μm cell strainer and centrifuged at a low speed (500rpm) to discard hepatocytes. The homogenate is washed twice and carefully layered in a double density gradient (17% bottom and 11.5% top) and centrifuged. A HSC rich layer is found at the top of the 11.5% gradient. The cells are collected, washed, counted and plated in tissue culture flasks to be expanded. It was observed during optimisation stages, that if the freshly isolated HSC were cultured in tissue culture plates (48-well or 24-well), they did not grow and proliferate. This could be because the plastic used in tissue culture plates differs from tissue culture flasks or because in plates the cells have restricted space to grow compared to a flask and this might effect their growth cycle. Approximately 7-10 days post culture in a tissue culture flask, HSC differentiate into myofibroblast like activated HSC.

To determine that the HSC isolated and grown, were purely HSC, their mRNA was extracted to run a RT PCR for levels of αSMA as is traditionally done (Figure 4.1a). Microscopically, the grown and cultured HSC cultured showed a characteristic ‘star-like’ myofibroblast phenotype (Figure 4.1b). The HSC were used between passage 2 and passage 5. Each batch of HSC was used at the same passage and days of culture for each individual experiment.

It is note worthy that all the liver resections used in this study were from patients undergoing liver surgery for removal of metastases. These patients might have undergone treatment against metastasis such as chemotherapy.

The influence of an individual's disease and/or treatment on their isolated HSC remains unknown. Whether this has affected the results presented in this chapter and the next chapter needs to be investigated further. It would be interesting to compare HSC isolated from the livers of non-cancerous patients, potentially from cadavers. However, this was not possible during the course of this study due to lack of ethics for obtaining such tissue. Also access to clinical data of these patients is necessary to determine if the differences in HSC of different donors was attributable to their prior treatment. We do not have access to this information at the moment, but this would be interesting to look at in the future.

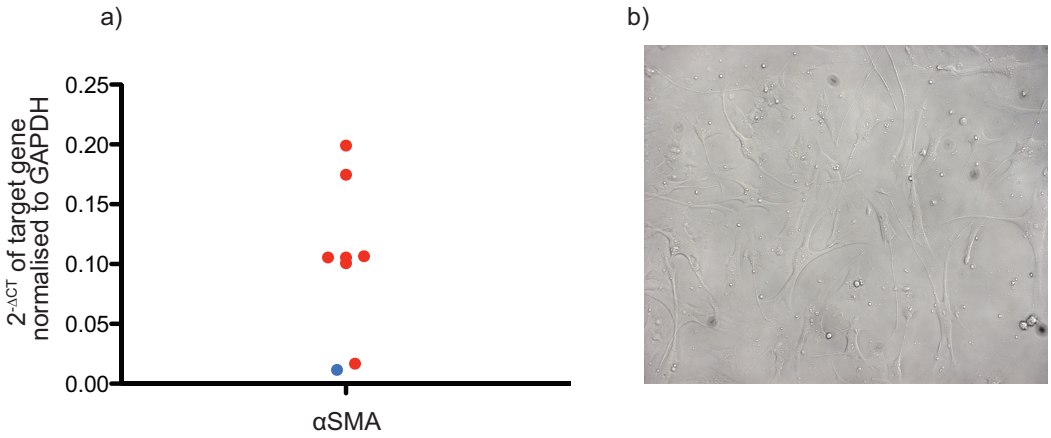


Figure 4-1: Baseline levels of αSMA and morphology HSC.

a) mRNA was extracted at the passage when primary HSC cells were used for experiments and was quantified using RT PCR to examine the levels of αSMA. mRNA levels of αSMA in different batches of HSC obtained from six different donors in red dots. mRNA levels of αSMA in LX2 cell line depicted in blue dot. b) Representative picture taken from light microscope of primary human HSC in culture at 10x magnification demonstrating their myofibroblast morphology.

4.2 Optimising read outs for apoptosis of HSC using flow cytometry

Classically, staining with Annexin V and 7-aminoactinomycin D (7-AAD) are used to quantify the degree of apoptosis in cells. Annexin V labeled with fluorescent probes can bind to phosphatidylserine and be detected using flow cytometry or fluorescent microscopy. In a normal healthy cell, phosphatidylserine is present on the cytoplasmic side of the cellular membrane and when the cell becomes apoptotic it is translocated to the outer part of the membrane where it can be detected using Annexin V. 7-AAD is a fluorescent molecule that can stain for nucleic acids and is therefore used for detection of dead cells with a permeabilised nucleus. This method of detection is very effective in determining dead or dying cells, however, it does not indicate whether the cell died via apoptosis, necrosis or necroptosis. In this study, we were interested in focusing on apoptosis of HSC; for this reason, we wanted the detection to be more focused towards staining of specific makers of apoptosis and so it was chosen to stain for caspases. A comparison was done between the traditional method of staining for Annexin V and 7-AAD with staining for pan-caspases and 7-AAD. To stain for pan-caspases, commercially available kit called Fluorescent labeled inhibitor of caspases (FLICA®) was used. These optimisations were done on both LX2 and primary human HSC. LX2 and primary human HSC were stained for apoptosis and death using AnnexinV/7-AAD and pan-caspase FLICA/7-AAD to optimise the read-out for apoptosis. As demonstrated in Figure 4.2 below, HSC being highly auto-fluorescent interfered with the fluorochromes of these markers, leading to improper staining detection. As can be seen in the plots, with both the staining in LX2 as well as primary HSC, a diagonal streak of population

can be observed. In such situations it is difficult to separate auto-fluorescence from real positive staining. Even though upon treatment with a positive control to induce death (camptothecin), there is an increment in the expression levels of respective markers, it is not a clear staining, requiring approximation in the gating strategy from which accurate data cannot be deduced.

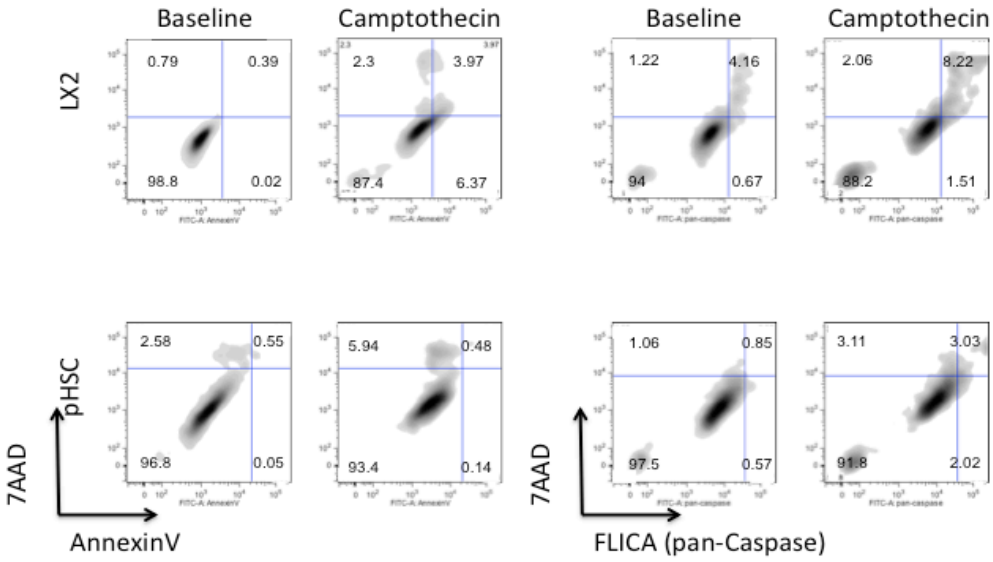


Figure 4-2: Apoptosis read out comparison of HSC.

LX2 (top panel) and primary HSC (pHSC) (bottom panel) and treated with camptothecin (8µg/ml) for 5 hours. LX2 and pHSC were then harvested and stained for either Annexin V and 7AAD or for FLICA (pan-caspase) and 7AAD and analysed using flow cytometry. Percentage expression of each marker is shown in each quadrant gate in the untreated condition and camptothecin treated condition.

Moving forward from this, to establish staining with a marker labeled with a fluorochrome that did not interfere or contribute to auto fluorescence. For this we stained LX2 and primary HSC with cleaved caspase 3 and Live/Dead cell viability stain. Cleaved caspase 3 was chosen because caspase 3 is the final caspase to be cleaved in the caspase cascade leading to apoptosis and therefore reflects the cells' commitment to undergo apoptosis. As demonstrated in Figure 4.3 below, successful staining for apoptosis was observed without any hindrance due to auto-fluorescence.

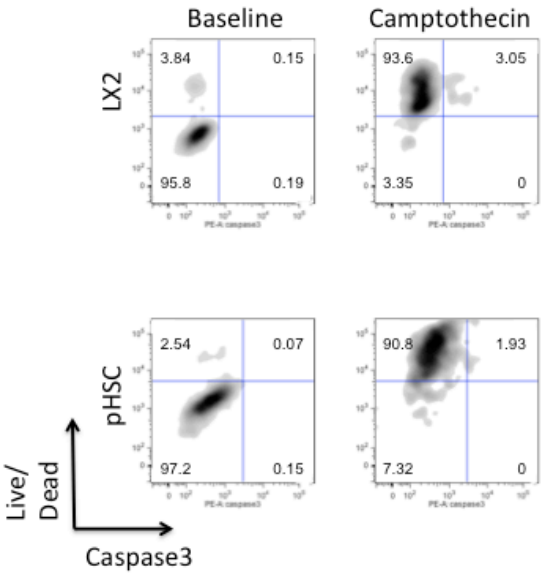


Figure 4-3: Apoptosis read out.

LX2 and primary HSC (pHSC) were treated with camptothecin (8µg/ml) for 5 hours. LX2 and pHSC were then harvested and stained for dead cell marker and cleaved caspase 3 and analysed using flow cytometry. FACS plots demonstrating a reliable readout of apoptosis on HSC. Percentage expression of each marker is shown in each quadrant gate in untreated condition and camptothecin treated condition.

In these optimization experiments, LX2 and primary HSC were both used to set up an effective readout for apoptosis. The results demonstrated that primary HSC can be used as reliable targets to study apoptosis and therefore, for the remaining project, primary cells were used to study the potential of HSC to undergo apoptosis.

4.3 Correcting for inter-donor variability of HSC to undergo apoptosis

Having established an effective readout for apoptosis of HSC, the next step was to optimise and setup the apoptosis assay. In this thesis, for all the assays primary human HSC have been studied. These primary human HSC have been isolated from healthy margins of metastatic liver tissue obtained from consented donors at the Royal Free Hospital. They were isolated from the liver tissue using enzymatic and mechanical digestion and then using three density gradients to obtain HSC. These HSC were then grown in tissue culture flasks and were spontaneously activated and expanded (for details, see Methods chapter). Aliquots of these were frozen in liquid nitrogen for long-term storage. The HSC were used in experiments between passage 2 and passage 5. In these assays, *SuperKiller* TRAIL was used as a positive control for apoptosis. *SuperKiller* TRAIL is a stable trimer of TRAIL that can induce apoptosis of target cells in a TRAIL-mediated manner. Before performing the experiments, to determine if there was variability in the degree of apoptosis in HSC from different HSC donors; HSC from two different donors were treated with positive control in the same experiment. As expected, inter-donor variability was observed in the potential of HSC from different donors to undergo apoptosis illustrated in Figure 4.4 below. To address this, the degree of apoptosis was determined by normalizing to that of the positive control in each experiment. For example, if the frequency of baseline apoptosis is 1 and that induced by positive control is 50, then positive control normalized baseline degree of apoptosis would be 1/50 x 100= 2%; assuming that the positive control reflects the maximum potential of the cells to undergo apoptosis is each individual experiment.

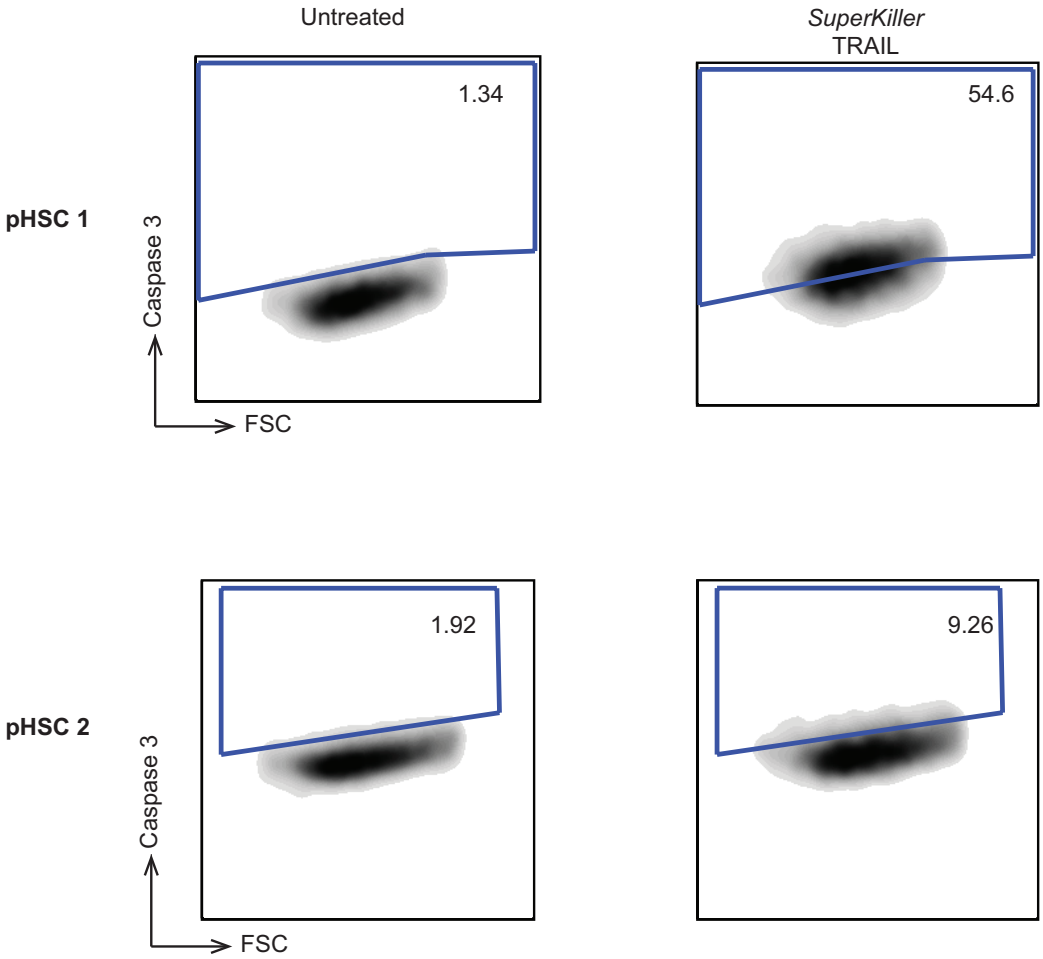


Figure 4-4: Variability in apoptosis potential of primary HSC from different donors.

FACs plots showing % expression of cleaved caspase 3 on primary human HSC (pHSC) from two different donors (pHSC1 and pHSC2) at baseline and on treatment with *SuperKiller* TRAIL (500ng/ml) for 5 hours.

4.4 Patient Cohort

Local and national ethical boards at Camden Primary Care Trust, University College Hospital, Royal London Hospital and Royal Free Hospital Trust approved this study. All participants of the study have given written, informed consent. All sample and data storage complies with the requirements of Data Protection Act 1998 and Human Tissue Act 2004. Peripheral blood was obtained from patients infected with CHB attending clinics at Mortimer Market Centre (Bloomsbury, London), University College London Hospital NHS Foundation Trust (Bloomsbury, London), Royal London Hospital, Barts Health NHS Trust (Whitechapel, London) and Royal Free London NHS Foundation Trust (Hampstead, London). All HBV infected patients were anti-HCV and anti-HIV antibody negative. The patients were stratified on the basis of their disease status: viral load (determined by real-time PCR), liver inflammation indicated by serum levels of alanine transaminase (ALT) and degree of fibrosis determined by Ishak stage and/or ELF test (Ishak score: 1-2 mild fibrosis, 3-4 moderate fibrosis, 5-6 cirrhosis; ELF test: ≤7.7 no/mild fibrosis, 7.7-9.8 moderate to severe fibrosis, >9.8 cirrhosis).

Clinical details of CHB patients along with their study codes are detailed in Table 4.1 below.

Pt ID	Fibrosis	HBeAg	Viral Load (IU/ml)	ALT (IU/L)	Gender	Age (yrs)	Treatment
Pt001	Moderate (ELF)	-	<2000	Not done	M	19	Untreated
Pt002	Moderate (ELF)	-	10,853	10	M	32	Untreated
Pt003	Not done	-	<2000	Not done	M	34	Untreated
Pt004	Cirrhosis (Biopsy)	-	29	Not done	M	49	Untreated
Pt005	Moderate (ELF)	-	Not done	Not done		36	Untreated
Pt006	Cirrhosis (Biopsy)	-	172	57	M	53	Untreated
Pt007	Moderate (Biopsy)	-	1,535	53	F	57	Untreated
Pt008	Moderate (Biopsy)	-	10,107	20	M	46	Untreated
Pt009	Cirrhotic (Biopsy)	-	Not detected	200	F	70	Untreated
Pt010	Mild (ELF)	-	Not detected	22	F	29	Untreated
Pt011	Severe (ELF)	-	110,000	53	M	44	Untreated
Pt012	Not done	-	Not detected	134	M	28	Untreated

Pt013	Moderate (ELF)	-	6,100	93	M	30	Untreated
Pt014	Moderate (ELF)	-	Not detected	84		19	Untreated
Pt015	Severe (ELF)	+	27,000,000	748	M	35	Untreated
Pt016	Not done	-	13,000	186	M	33	Untreated
Pt017	Moderate (Biopsy)	-	1,150	114		48	Untreated
Pt018	Moderate (ELF)	+	73,164,142	172	F	25	Untreated
Pt019	Not done	-	110	18	F	60	Untreated
Pt020	Mild (ELF)	-	16,000	47	F	35	Untreated
Pt021	Mild (ELF)	-	1,800	18	F	28	Untreated
Pt022	Moderate (ELF)	Not done	Not detected	27	M	33	Untreated
Pt023	Moderate (ELF)	Not done	Not detected	24	F	27	Untreated
Pt024	Moderate (ELF)	-	7,066	26	F	40	Untreated
Pt025	Mild (ELF)	-	421	58	M	36	Untreated
Pt026	Severe	+	32,000	160	M	59	Untreated

	(Biopsy)						
Pt027	Not done	-	13,000	186	M	33	Untreated
Pt028	Moderate (ELF)	-	1,127,317	189	F	52	Untreated
Pt029	None (Biopsy)	-	347	25	F	43	Untreated
Pt030	Moderate (Biopsy)	-	1150	114		48	Untreated
Pt031	Not done	-	2,832	41	F	33	Untreated
Pt032	Not done	+	100,000,000	233	M	32	Untreated
Pt033	Not done	-	9,400	25	F	60	Untreated
Pt034	Not done		430	21	F	56	Untreated
Pt035	Cirrhotic (Biopsy)	-	Not detected	51	M	36	Untreated
Pt036	Not done	-	2,200	24	F	45	Untreated
Pt037	Not done	-	1,500	19	F	31	Untreated
Pt038	Not done	-	Not done	19	F	34	Untreated
Pt039	Not done	+	14,911,605	74	M	33	Untreated

Pt040	Not done	-	Not done	Not done	F	27	Untreated
Pt041	Not done	-	1,100	22	F	41	Untreated
Pt042	Not done	-	Not detected	22	M	36	Untreated
Pt043	Not done	-	180	20	F	22	Untreated
Pt044	Not done	-	220	41	F	36	Untreated
Pt045	Not done	-	1000	34	M	40	Untreated
Pt046	Not done	-	Not detected	86	M		Untreated
Pt047	Not done	-	Not detected	Not done	M	29	Untreated
Pt048	Not done	+	2000	Not done	M	30	Untreated
Pt049	Not done	+	Not done	Not done		38	Untreated
Pt050	None (Biopsy)	-	4850	26	F	35	Untreated
Pt051	Not done	-	Not detected	39	M	41	Untreated
Pt052	Mild (Biopsy)	-	5440	47	M	44	Untreated
Pt053	Mild	-	33,500	19	F	42	Untreated

	(Biopsy)						
Pt054	Not done	-	2,700	25	M	43	Untreated

Table 4-1: Details of CHB patients whose PBMC were used in this study.

First column is the patient sample code, second column is the fibrosis score of patients, third column gives the HBeAg status of the patient, fourth column is the HBV DNA (viral load) of the patients (IU/ml), fifth column gives alanine aminotransferase (ALT) levels of the patient in IU/L, sixth column indicates the gender of the patient (Female=F, Male=M), seventh column indicates the age of the patient at the time of sample collection in years and the eighth column indicates the treatment status of the patients.

Table 4.2 below gives the details of the healthy donors that consented and participated in this study.

Pt Id	Gender	Age
HD001	M	31
HD002	F	26
HD003	M	29
HD004	F	34
HD005	M	25

HD006	F	36
HD007	M	23
HD008	F	38
HD009	M	31
HD010	F	44
HD011	F	32
HD012	F	22
HD013	F	32
HD014	F	32
HD015	M	35
HD016	M	32
HD017	F	33
HD018	M	27
HD019	M	24

HD020	F	26

Table 4-2: Details of healthy donors whose PBMC were used in this study.

First column gives the sample code, second column gives the gender of the participant (F=female, M=male) and the third column gives the age of the participant in years at the time when sample was collected.

4.5 Developing an assay to study the role of NK cells in HSC apoptosis

Having normalized the read-out for apoptosis of HSC, the experimental design was created. The HSC plated on 48-well tissue culture plates were co-cultured with NK cells from CHB patients or healthy donors. Post co-culture, the apoptosis of HSC was determined by staining them for cleaved caspase-3 and analysed using flow cytometry. NK cells were purified from whole PBMC using a magnetic bead NK cell isolation kit. As can be seen in Figure 4.5 below, very clean purities of NK cells were obtained, determined by staining for CD56+ CD3- cells and analysed using flow cytometry.

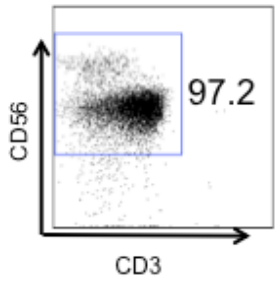


Figure 4-5: NK cell purity post magnetic bead isolation.
FACS plot demonstrating the levels of purity post magnetic bead isolation of NK cells by gating on CD56+ CD3- cell population. Doublets and dead cells have been excluded in previous gates.

NK cells isolated from PBMC of CHB patients and healthy donors were co-cultured with already plated primary HSC for five hours as illustrated in figure 4.6 below.

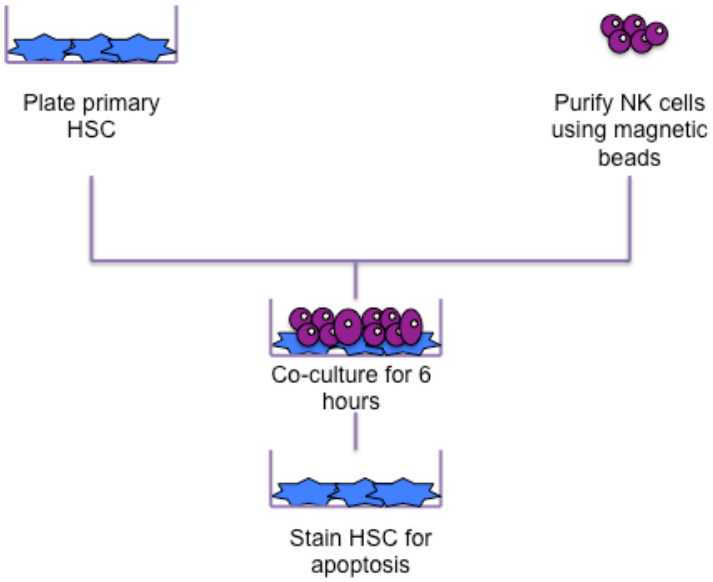


Figure 4-6: Experimental design.

Experimental design to study NK cell-HSC interaction and examine the apoptosis-inducing potential of NK cells against HSC. Primary human HSC were plated 24 hours prior to setting up co-culture experiments at a density of 10,000 cells per well. NK cells were purified from whole PBMC using a magnetic bead NK cell isolation kit and left in culture overnight. NK cells and HSC were co-cultured for 5 hours. At the end of co-culture, HSC were harvested using Trypsin/EDTA and stained for Dead cell marker and cleaved caspase 3 to study apoptosis. The data was analysed using flow cytometry. *Superkiller* TRAIL (500ng/ml) was used as a positive control for apoptosis.

4.6 NK cells have a variable and limited potential to kill HSC

Having optimised and established the conditions to effectively measure apoptosis of HSC, the co-culture experiments were carried out measuring apoptosis of HSC at baseline, with a positive control and in co-culture with NK cells purified from PBMC of a cohort of 18 healthy and 59 CHB donors. As demonstrated in Figure 4.7a below, NK cells from some donors were able to induce a degree of apoptosis of HSC. Figure 4.7b shows the cumulative data of induction of apoptosis in HSC by NK cells of CHB patients and healthy

individuals. These data have been normalized to apoptosis induced by the positive control (*SuperKiller* TRAIL, 500ng/ml) in each individual experiment. This is done by assuming that the positive control reflects the maximum (100%) potential of the HSC to undergo apoptosis is each individual experiment, the cleaved caspase 3 levels of HSC at baseline and post co-culture with NK cells are calculated in relation to positive control. As can be seen in Figure 4.7, NK cells from CHB patients can significantly induce apoptosis of HSC. However for the whole cohort, this is not significantly higher than that induced by NK cells from the cohort of healthy individuals. A large spread in the killing potential of NK cells of CHB patients and healthy donors can be observed.

NK cells from some healthy donors and CHB patients have a better potential to induce apoptosis compared to others. NK cells from the majority of the donors are unable to induce effective apoptosis of HSC. These data indicate that NK cells have a variable and limited potential to induce apoptosis of HSC.

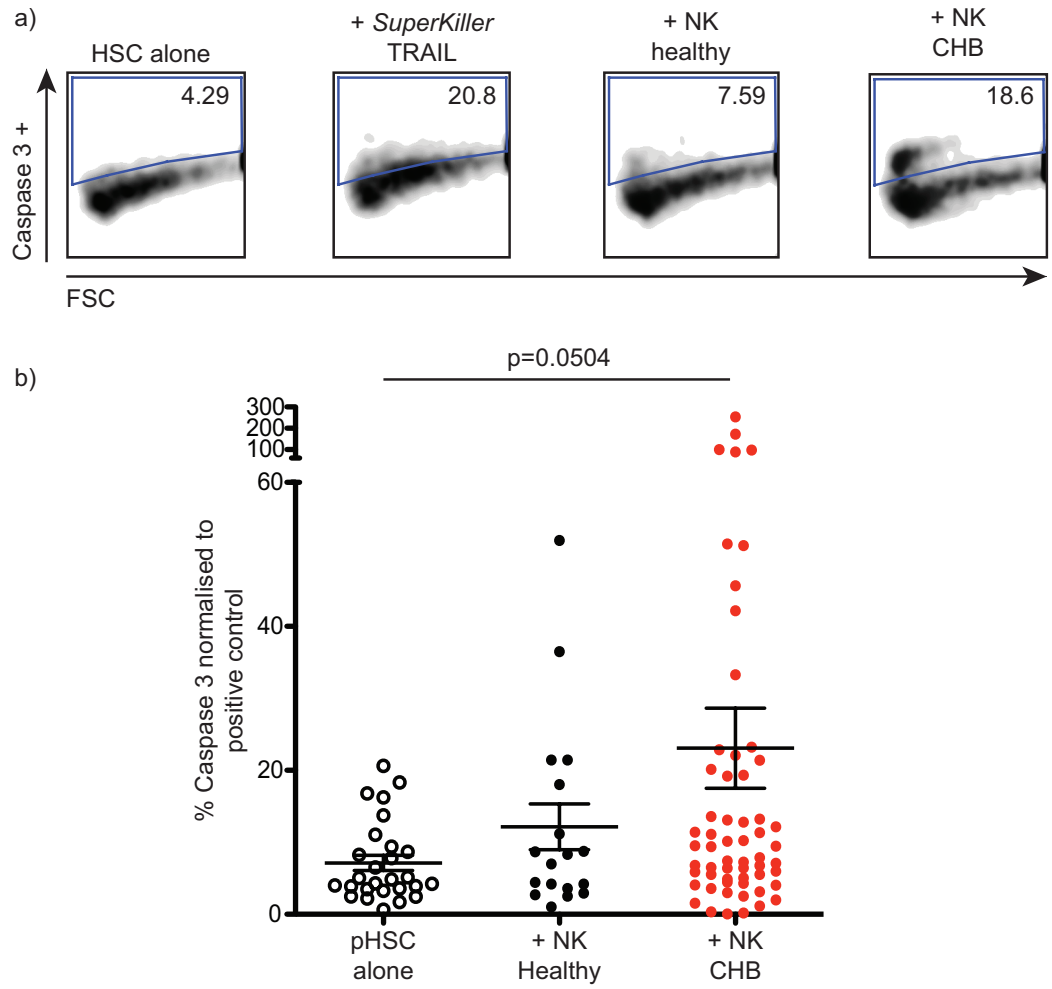


Figure 4-7: NK cells have a variable capacity to trigger apoptosis in HSC. Human primary HSC were co-cultured with NK cells from healthy donors and CHB patients for 5 hours. Treatment of HSC with *Superkiller* TRAIL (500ng/ml) for the same duration as the co-culture was used as the positive control. a) Representative FACS plot demonstrating the capacity of NK cells from a healthy donor and patient with CHB, compared to *SuperKiller* TRAIL, to induce cleaved caspase 3 expression in HSC. b) Summary graph showing % of cleaved caspase 3 normalised to positive control in primary HSC (pHSC) alone, after co-culture with NK cells from healthy donors and after co-culture with NK cells from CHB patients. (p=0.0504, Kruskal-Wallis ANOVA).

4.7 Capacity of NK cells to kill HSC varies according to patient CHB disease status

To address this variable potential of NK cells to kill HSC, the CHB patients were stratified on the basis of their HBeAg status, viral load, ALT and fibrosis stage. As previously mentioned in the Introduction section of this thesis, the various parameters that determine disease status may influence the fibrosis status of patients. The patients were stratified on the basis of their disease status: viral load (determined by real-time PCR), liver inflammation indicated by serum levels of alanine transaminase (ALT) and degree of fibrosis determined by Ishak stage (ranked by a histopathologist as part of routine clinical diagnosis) and/or ELF test (Ishak score: 1-2 mild fibrosis, 3-4 moderate fibrosis; ELF test: 0-7.7 no/mild fibrosis, 7.7-9.8 moderate to severe fibrosis). To understand this more finely, a table showing the various stages of each patient is given in Table 3.1 and following that is the stratified data showing the influence of the disease status on the ability of NK cells to induce apoptosis of HSC in Figure 4.8.

The cumulative results of CHB disease stratification are illustrated below in Figure 4.8.

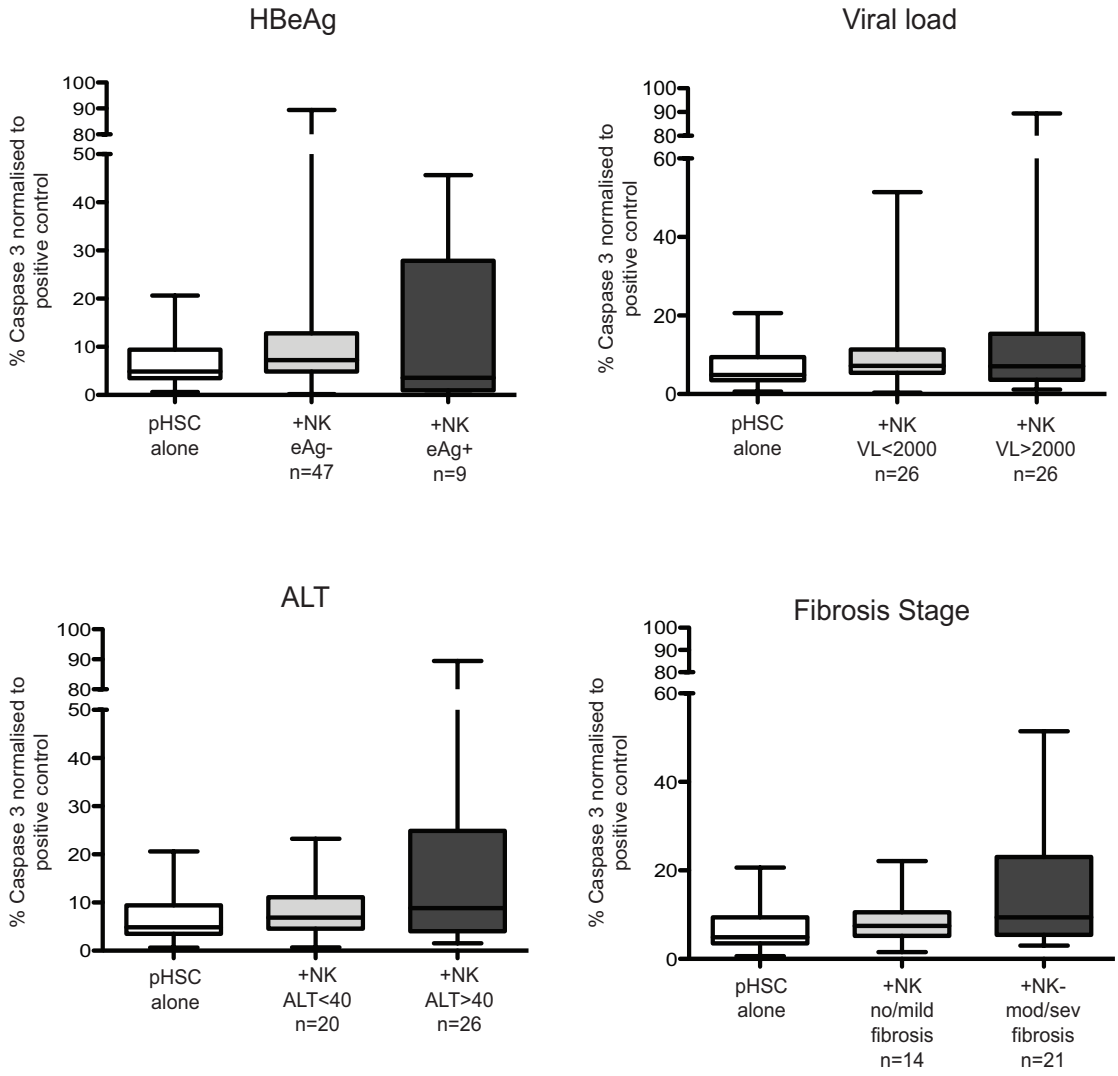


Figure 4-8: Stratification of CHB patients and their potential to kill HSC. Human primary HSC were co-cultured with NK cells from CHB patients for 5 hours. Treatment of HSC with *Superkiller* TRAIL (500ng/ml) for the same duration as the co-culture was used as the positive control. % of cleaved caspase 3+ primary human HSC (pHSC) post co-culture normalized to positive control. Cumulative plots showing capacity of NK cells of CHB patients to kill HSC, dividing patients on the basis of HBeAg (p=0.0695, Kruskal-Wallis ANOVA), HBV viral load (p=0.1935, Kruskal-Wallis ANOVA), ALT (p=0.1266, Kruskal-Wallis ANOVA) and stage of fibrosis (p=0.0635, Kruskal-Wallis ANOVA).

These data indicate that the HBeAg status, viral load, ALT levels and fibrosis stage of the patients does not influence the killing potential of their NK cells statistically. However, patients with ALT > 40 have a trend towards an enhanced potential of their NK cells to kill HSC. Previous work has demonstrated that NK cells of CHB patients with elevated levels of ALT have high levels of TRAIL expression (Dunn et al., 2007; Peppa et al., 2010). NK cells of CHB patients with moderate to severe fibrosis also exhibit a trend towards improved capacity to kill HSC however this is not statistically significant compared to NK cells of patients with no or mild fibrosis.

Next we wanted to investigate whether the gender of the patients influenced the capacity of their NK cells to induce apoptosis of HSC. For this, the patients from the cumulative graph in Figure 4.7 were divided on the basis of their gender. As can be seen in Figure 4.9 below, the gender of the patients had no significant effect on the potential of their NK cells to induce apoptosis of HSC.

Furthermore, we also wanted to examine if the age of the patients had an impact on the apoptosis inducing capability of NK cells. To study this, the age of the CHB patient at the time of sampling was correlated with their potential to trigger apoptosis of HSC. Figure 4.10 demonstrates that in the current cohort, no statistical correlation exists between these factors.

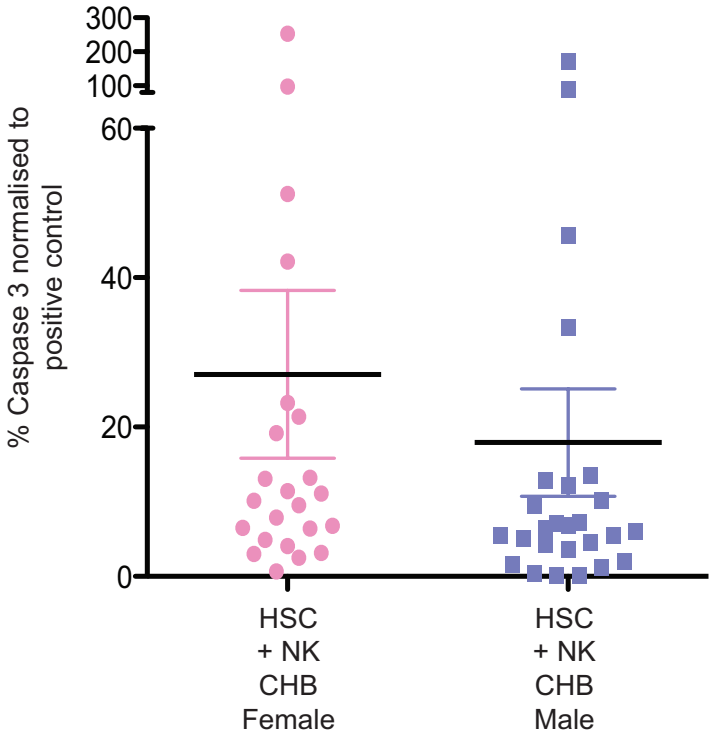


Figure 4-9: Segregation of CHB patients on the basis of gender and their capacity to kill HSC.

Human primary HSC were co-cultured with NK cells from CHB patients for 5 hours. Treatment of HSC with *Superkiller* TRAIL (500ng/ml) for the same duration as the co-culture was used as the positive control. % of cleaved caspase 3+ primary human HSC (pHSC) post co-culture is normalized to positive control. Cumulative graph showing the capacity of NK cells of female CHB patients and male CHB patients to induce apoptosis of HSC. No statistical difference was found between the two (p=0.1205, Mann-Whitney U test).

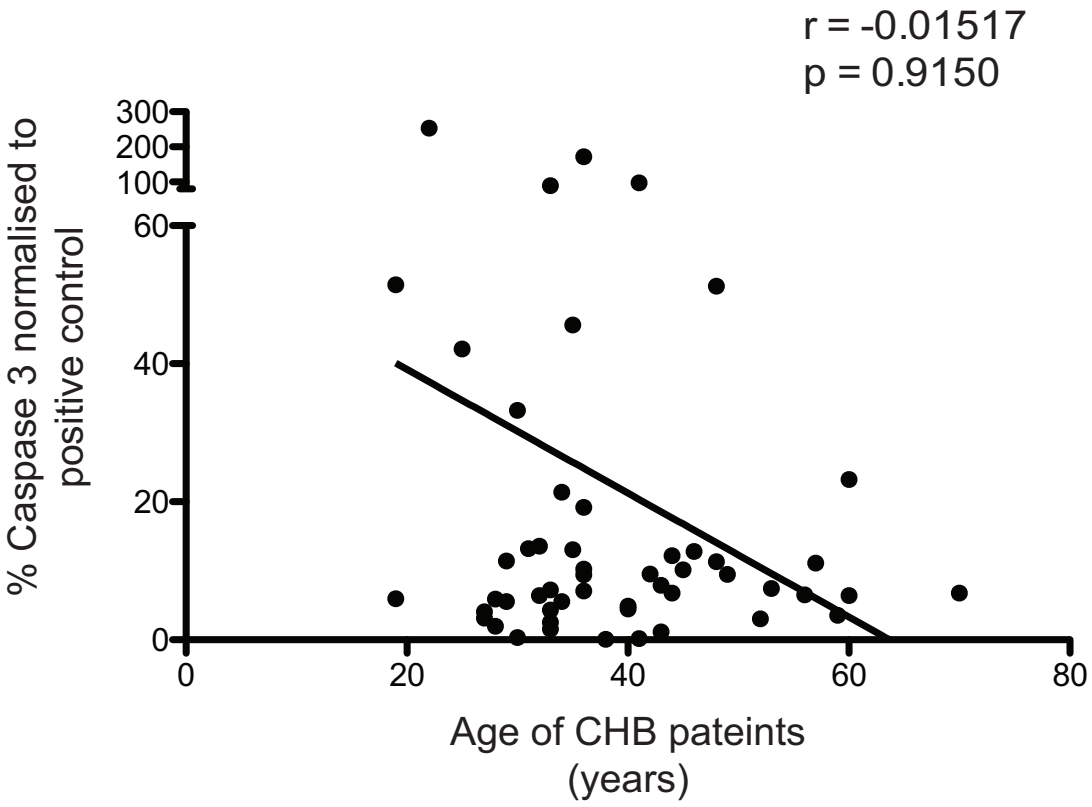


Figure 4-10: No correlation between age of CHB patients and their capacity to induce apoptosis of HSC.

Human primary HSC were co-cultured with NK cells from CHB patients for 5 hours. Treatment of HSC with *Superkiller* TRAIL (500ng/ml) for the same duration as the co-culture was used as the positive control. % of cleaved caspase 3+ primary human HSC (pHSC) post co-culture is normalized to positive control. Correlation graph showing the age of CHB patients in years (x axis) against their capacity to induce apoptosis of HSC (y axis). No statistical significance found.

4.8 Effects of antiviral treatment on NK cell potential to kill HSC

Next we wanted to examine the effect of anti-viral treatment in patients with CHB on their NK cells to determine if this influenced their potential to kill HSC. The patient cohort used for this analysis and their clinical details are enlisted in Table 4.3 below. As can be seen in the cumulative plot below (Figure 4.11), NK cells of patients on NUC therapy were unable to induce apoptosis of HSC. However, there was no statistical difference between the potential of NK cells from untreated CHB patients and that of CHB patients on NUC therapy. It is worth noting that the cohort size of CHB patients on treatment is much smaller compared to untreated patients. These findings are in line with previously published data from our group showing that NK cells of CHB patients on NUC treatment are less activated and express less TRAIL compared to those of untreated patients (Peppa et al., 2010). Phenotyping NK cells for activation and TRAIL expression from patients used in these experiments complemented previously published data. Plausibly due to their less activated phenotype, these NK cells from CHB patients on NUCs are unable to trigger apoptosis of HSC.

Pt Id	HBeAg	Viral load (IU/ml)	ALT (IU/ml)	Treatment	Fibrosis status	Age (yrs)
Pt1	+	29,786	61	Lam+Adv	Mild	48
Pt2	-	Not detected	17	Adv	Moderate	64
Pt3	-	16,000	47	Adv	Mild	40
Pt4	+	Not detected	42	Lam+Adv	Moderate	59
Pt5	+	Not detected	36	Ten	Not done	62
Pt6	-	60 $x10^6$	126	Lam	Not done	79
Pt7	+	179	38	Ten	Not done	31
Pt8	-	Not detected	18	Ten	Not done	48
Pt9	-	Not detected	18	Ten	Not done	52

Table 4-3: Details of CHB patients on NUC therapy whose PBMC were used in this study.

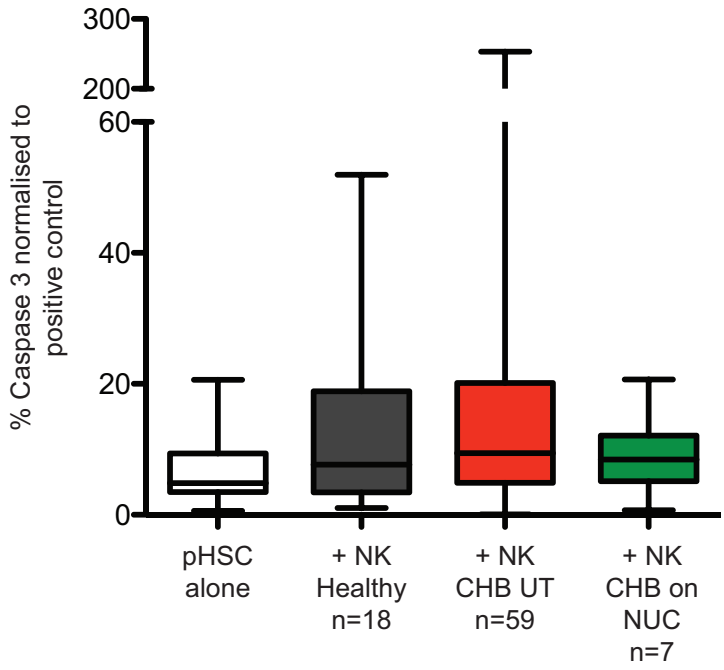


Figure 4-11: NK cells from CHB patients on NUCs do not show any difference in their capacity to induce apoptosis of HSC compared to baseline apoptosis of HSC.

Graph showing cumulative data of % of cleaved caspase 3 levels normalized to positive control (*SuperKiller* TRAIL 500ng/ml) on primary HSC (pHSC) after co-culture with NK cells from healthy donors, untreated (UT) CHB patients and CHB patients on NUCs using flow cytometry. (p=0.1052, Kruskal-Wallis ANOVA).

Following on from this, we wanted to examine if activating NK cells influenced their potential to kill HSC.

4.9 NK cells activated with IFNα have an augmented potential to kill HSC

NK cells can be activated in the presence of cytokines such as IFN-α as mentioned before. Previous work from our group and others has demonstrated that NK cells are activated on *in vitro* and *in vivo* treatment with IFN-α (Micco et al., 2013; Walzer et al., 2005). IFN-α has also been demonstrated to have a direct anti-fibrotic effect by reducing the proliferative potential of HSC in *in vitro* experiments with LX2 in a dose dependent manner (Ogawa et al., 2009), however, this needs to be investigated in further detail. In this study, to examine whether activated NK cells had an enhanced potential to induce apoptosis of stellate cells, purified NK cells were activated *in vitro* with recombinant IFN-α (rIFN-α) (10^5 U/ml) overnight and washed before being co-cultured with HSC to determine apoptosis of HSC, as described in Materials and Methods. This working concentration of rIFN-α was chosen as it has been previously described that this is the approximate concentration of IFN-α found in the circulation of CHB patients on peg-IFN-α therapy (Dunn et al., 2007). We observed that upon activation with IFN-α, NK cells had an improved capacity to kill HSC as illustrated in representative FACS plot in Figure 3.12a. The cumulative graphs of these data in Figure 3.12b show a significant increase in the killing capacity of NK cells activated by IFN-α over untreated NK cells. This enhanced capacity of NK cells to kill HSC was observed both in NK cells from CHB patients and healthy donors, which implies that this is not a CHB specific effect and IFN-α activated NK cells in general exhibited a better anti-fibrotic capacity to kill HSC.

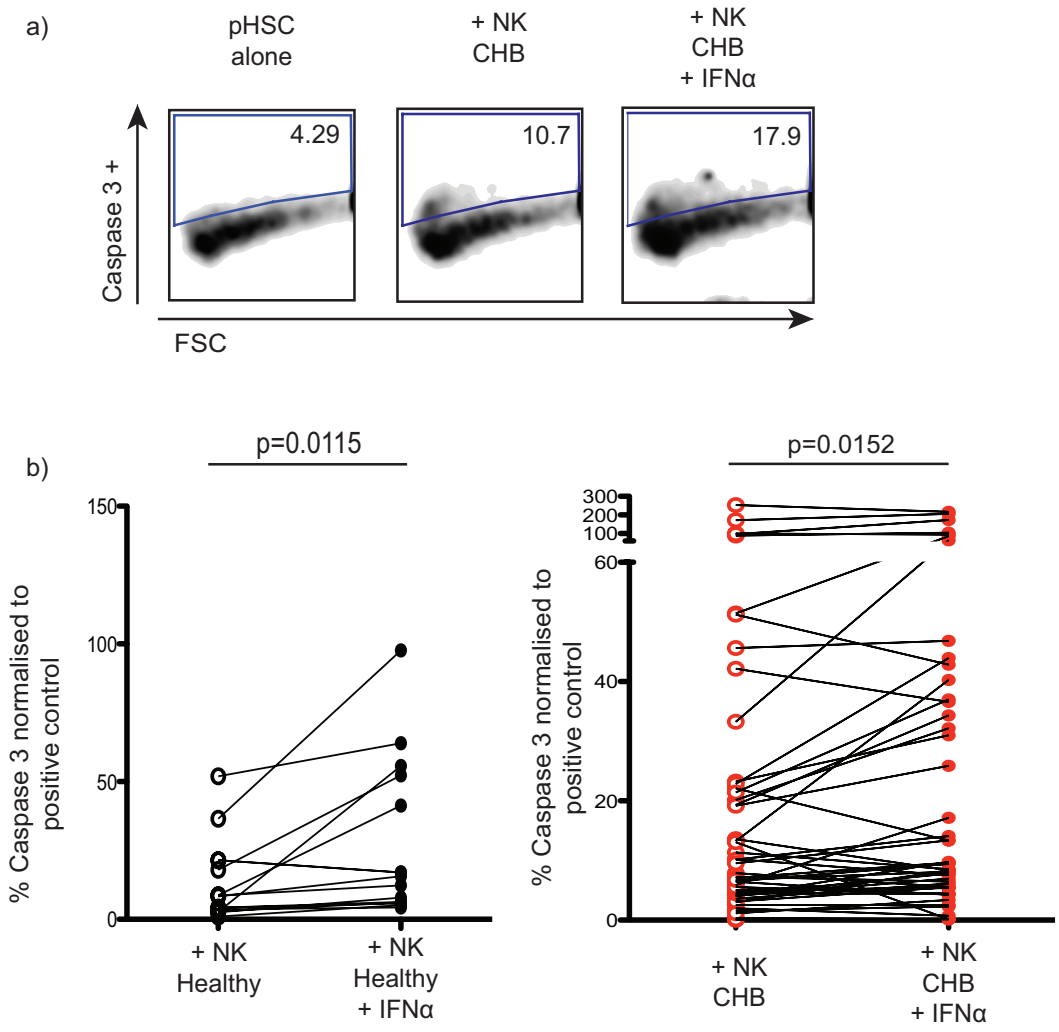


Figure 4-12: Effect of activated NK cells on HSC apoptosis.

NK cells from healthy donors and CHB patients were left untreated or were treated with rIFNα (10^5 U/ml) overnight and co-cultured with primary human HSC (pHSC) for 5 hours. % expression of cleaved caspase 3+ HSC was determined using flow cytometry. a) Representative FACS plot showing improved capacity of NK cells treated with IFNα to kill HSC and b) cumulative plots of impact of IFN-a on the capacity of NK cells from healthy donors (p=0.0115, Wilcoxon paired test) and CHB patients (p=0.0152, Wilcoxon paired test) to kill HSC.

These data demonstrated that IFN-α increased the potential of NK cells to kill HSC. These data led us to probe the pathway through which NK cells could be killing HSC.

4.10 Examining whether peripheral NK cells reflect the intra-hepatic NK cells in the context of their interaction with HSC

As has been described in the Introduction section, NK cells in the liver comprise around 40% of the lymphocyte population compared to 8-15% in the periphery (Cooper et al., 2001). They are not only more concentrated in the liver but also phenotypically different, in that the liver contains a greater proportion of $CD56^{bright}$ NK cells compared to the periphery. We have previously shown that intrahapetic NK cells are more activated and express higher levels of TRAIL than those in the circulation (Dunn et al., 2007). This phenotype of liver NK cells has interesting parallels with recent high profile publications revealing a separate lineage of liver-resident NK cells in murine models with a different transcriptional profile and high levels of TRAIL expression (Daussy et al., 2014; Peng et al., 2013). It would be important therefore to examine the interaction between liver NK cells and HSC, since this may represent a unique physiological function for liver-lineage NK cells. Some preliminary experiments were conducted using intrahepatic lymphocytes (IHL) extracted from surplus biopsy tissue from CHB patients and perfusates from livers of healthy organ donors. As illustrated in Figure 4.13, IHL from CHB patients in one case showed a better potential to kill HSC compared to IHL from healthy individuals. It is noteworthy that these are very preliminary results and were obtained from only two samples from both

healthy and CHB patients. It is impossible to conclude anything from these few samples, however, it would be interesting to investigate the apoptosis inducing potential of liver NK cells from CHB patients compared to their circulating counterparts and compared to intrahepatic NK cells from healthy controls.

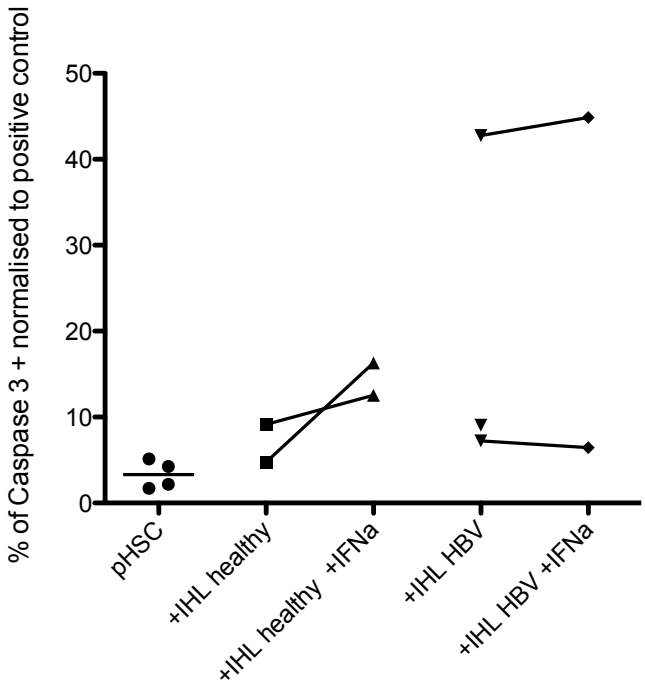


Figure 4-13: HSC apoptosis induced by IHL from healthy and CHB donors.

Cleaved caspase 3 levels of primary HSC (pHSC) when co-cultured with intra-hepatic lymphocytes (IHL) isolated from healthy donor livers and liver biopsies from CHB with and without *in vitro* treatment with IFN-α. These data were collected using flow cytometry.

4.11 IFN-α upregulates expression levels of TRAIL on NK cells

To investigate the pathway through which NK cells trigger apoptosis in HSC that was augmented by IFN-α, both treated (with rIFN-α) and untreated NK cells were phenotyped from all patients and healthy donors. This was done at the time of co-culture. Out of all NK markers that were stained for, the death ligand TRAIL was the only marker that was significantly upregulated on NK cells on *in vitro* treatment with IFN-α. The summary data for this are shown in Figure 4.14. These data suggested that the mechanism through which NK cells treated with rIFN-α have an improved capacity to kill HSC is the TRAIL pathway.

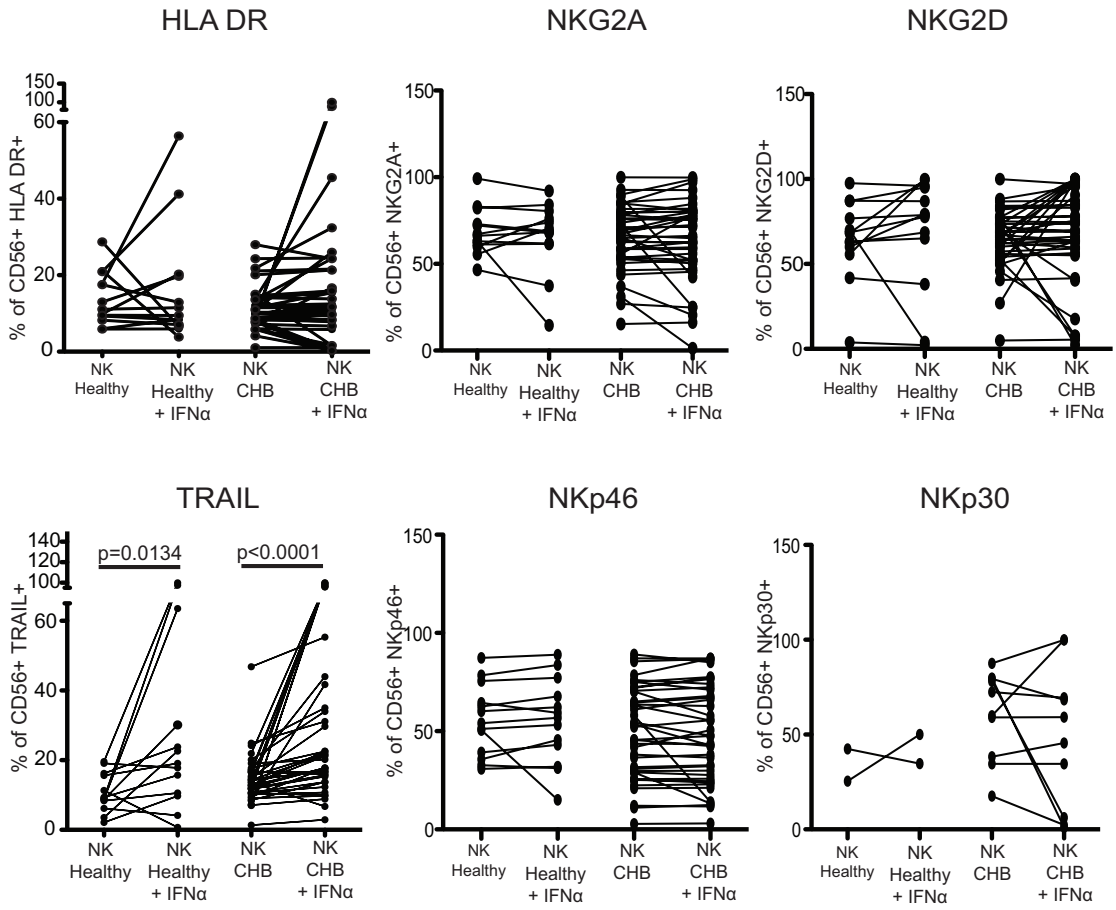


Figure 4-14: Effect of *in vitro* rIFN-α stimulation on NK cells.

NK cells from healthy donors and CHB patients were either left untreated treated with rIFNα (10^5 U/ml) overnight and stained for the specified markers. No statistical significance was observed on NK cells of healthy donors and/or CHB patients post *in* vitro treatment with rIFNα in the expression levels of HLA DR (p=0.7354, p=0.1495 Wilcoxon paired test) , NKG2A (p=0.6848, p=0.5163 Wilcoxon paired test), NKG2D (p=0.1677, p=0.1698 Wilcoxon paired test), NKp46 (p=0.1619, p=1.000 Wilcoxon paired test) and NKp30 (p=0.4961, Wilcoxon paired test) on NK cells from healthy donors and CHB patients. Statistically significant differences were found in the levels of TRAIL (p=0.0134, p<0.0001 Wilcoxon paired test) on NK cells from healthy donors and CHB patients.

TRAIL is a death ligand belonging to the tumor necrosis family superfamily, which can induce apoptosis of target cells expressing the relevant TRAIL-receptor. Significant upregulation of TRAIL on NK cells irrespective of the disease status, and not any other NK cell activatory receptor, suggested that TRAIL might be the dominant pathway via which NK cells exhibited improved potential to induce apoptosis of HSC upon *in vitro* pre-treatment and activation with rIFN-α.

4.12 HSC undergo TRAIL mediated apoptosis

As a proof of principle to test whether TRAIL was an effective mechanism through which HSC could undergo apoptosis, a lentiviral system was used to knock-down the expression of TRAIL-R2 on HSC. TRAIL can interact with TRAIL-R1 and TRAIL-R2 to induce apoptosis. HSC have been previously shown to express TRAIL-R2 (Taimr et al., 2003). In collaboration with Itziar Otano in the group, short hairpin (sh) RNA delivered by lentiviral vectors were developed to knock-down the expression of TRAIL-R2 on HSC (Otano, I unpublished). LX2 were used for this setup. Using this system, the expression of TRAIL-R2 was effectively knocked down from LX2 using shTRAIL-R2 compared to control sh (sh CTR) as illustrated in Figure 4.15 below.

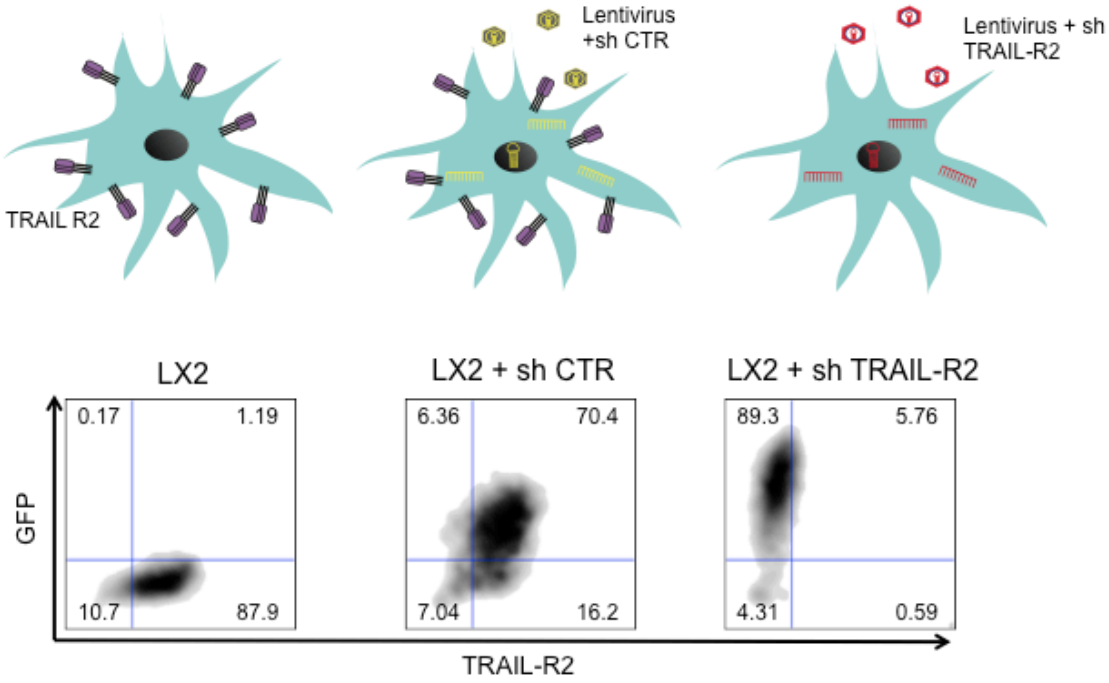


Figure 4-15: Knock-down of TRAIL-R2 on HSC using lentiviral vectors. LX2 were transduced using lentivirus containing control short hairpin (sh CTR) or short hairpin against TRAIL-R2 (sh TRAIL-R2) at an MOI of 40. The plasmids containing short hairpin also had GFP to determine effective transduction of cells. Diagramatic illustration (top panel) and FACS plot showing effective transduction of LX2 using a lentiviral vector to deliver shCTR and shTRAIL-R2 by looking at the expression of GFP and the levels of TRAIL-R2.

Next, to determine if knocking down TRAIL-R2 influenced apoptosis of HSC, LX2 were treated with *Superkiller* TRAIL and as shown in Figure 4.16, the majority of untransduced LX2 and those transduced with shCTR underwent apoptosis in a TRAIL-dependent manner. However, LX2 lacking TRAIL-R2 owing to knock-down of the receptor were protected from TRAIL mediated killing.

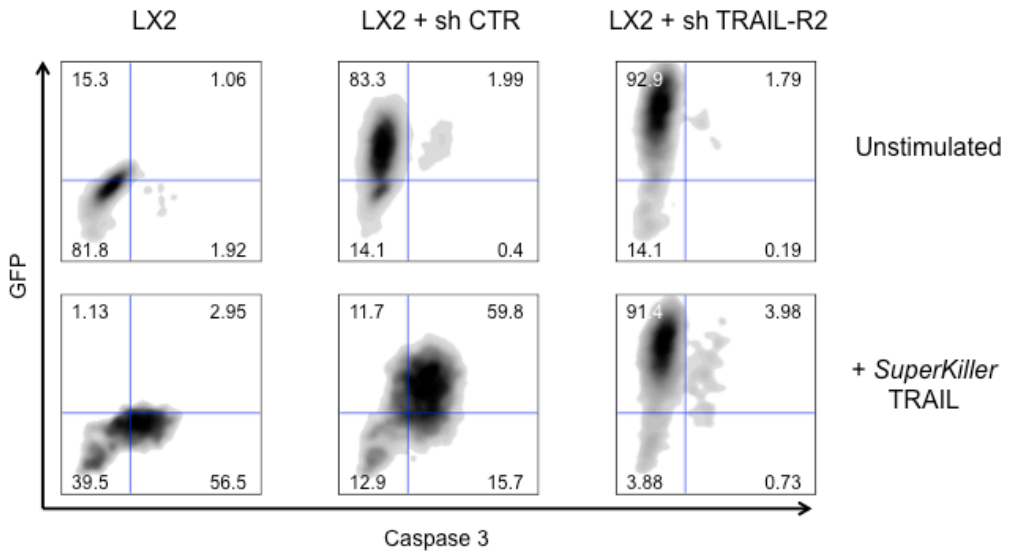


Figure 4-16: Rescue of TRAIL mediated apoptosis of HSC by knocking down TRAIL-R2.

LX2 transduced using lentivirus containing control short hairpin (sh CTR) or short hairpin against TRAIL-R2 (sh TRAIL-R2) at an MOI of 40. The plasmids containing short hairpin also had GFP to determine effective transduction of cells. Un-transduced and transduced LX2 were treated with *SuperKiller* TRAIL (500ng/ml) for five hours and their degree of apoptosis was examined by staining for cleaved caspase 3 and analysed using flow cytometry. FACS plots showing levels of cleaved caspase 3 on untreated un-transduced HSC, HSC transduced with sh CTR and HSC transduced with sh TRAIL R-2 (top panel). Bottom panel showing cleaved caspase 3 levels of un-transduced HSC, HSC transduced with sh CTR and HSC transduced with sh TRAIL R-2 upon treatment with *SuperKiller* TRAIL.

These data indicate that high levels of TRAIL can induce apoptosis of HSC. However, it is noteworthy that even with very high levels of TRAIL, as in this experiment, around 40% of HSC were not susceptible to apoptosis. This suggests that there could be other underlying mechanisms that allow HSC to escape apoptosis.

4.13 Blocking TRAIL on NK cells has no effect on their potential to kill HSC

To investigate the role of TRAIL in relation to NK cell killing of HSC, TRAIL was blocked on NK cells using neutralising antibodies against TRAIL before co-culturing them with HSC. We found that blocking TRAIL was able to reduce NK cell killing in some patients but not in others, such that it had no overall effect on the killing potential of NK cells from the whole cohort. These data have been summarized in Figure 4.14. As can be seen in Figure 4.17a, TRAIL blockade did reduce the NK cell-mediated induction of cleaved caspase 3 on HSC in some cases while in other cases TRAIL blockade induced HSC apoptosis. The cumulative data for this are shown in Figure 4.17b.

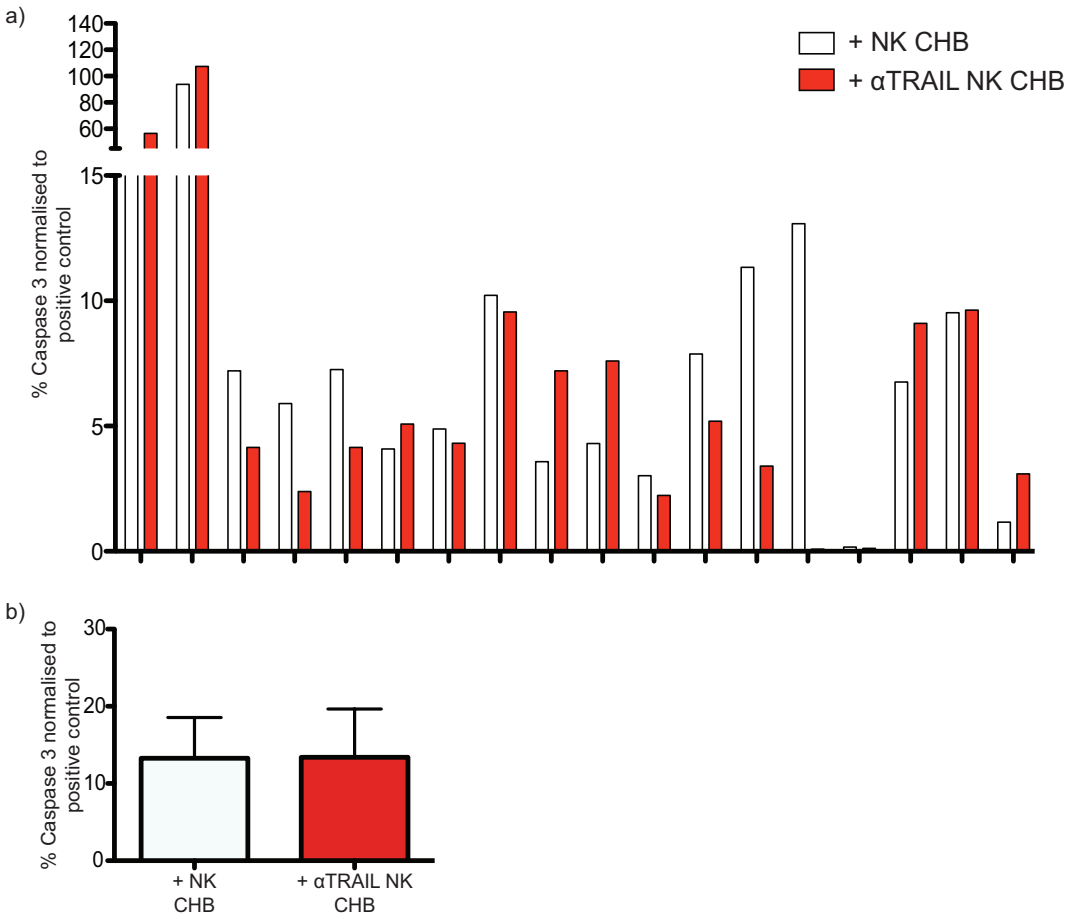


Figure 4-17: TRAIL blockade overall does not influence the apoptosis inducing potential of NK cells on HSC.

TRAIL was blocked on NK cells from CHB patients using neutralising antibodies against TRAIL (1µg/ml) prior to co-culture with primary human HSC. Above data depicts the potential of these NK cells to induce apoptosis in primary HSC normalized to positive control. a) Data from individual patients showing the effect of NK cells killing HSC before (white bar) and after TRAIL blockade (red bar). b) Cumulative plot showing no overall difference in the effect of blocking TRAIL on NK cells before co-culturing them with HSC (p=0.9306, Wilcoxon paired test) and their potential to trigger apoptosis of HSC.

Next we wanted to examine the effect of clinical status of CHB patients on the potential of their NK cells to kill HSC post blocking TRAIL on their surface. For this, the CHB patients from Figure 4.17 were segregated on the basis of their HBeAg, viral load, ALT levels and stage of fibrosis (determined using IIshak fibrosis stage or ELF test). Figure 4.18 demonstrates that in this cohort, the clinical parameters of the patients studied, do not influence the capacity of NK cells (post TRAIL blockade) to trigger apoptosis of HSC.

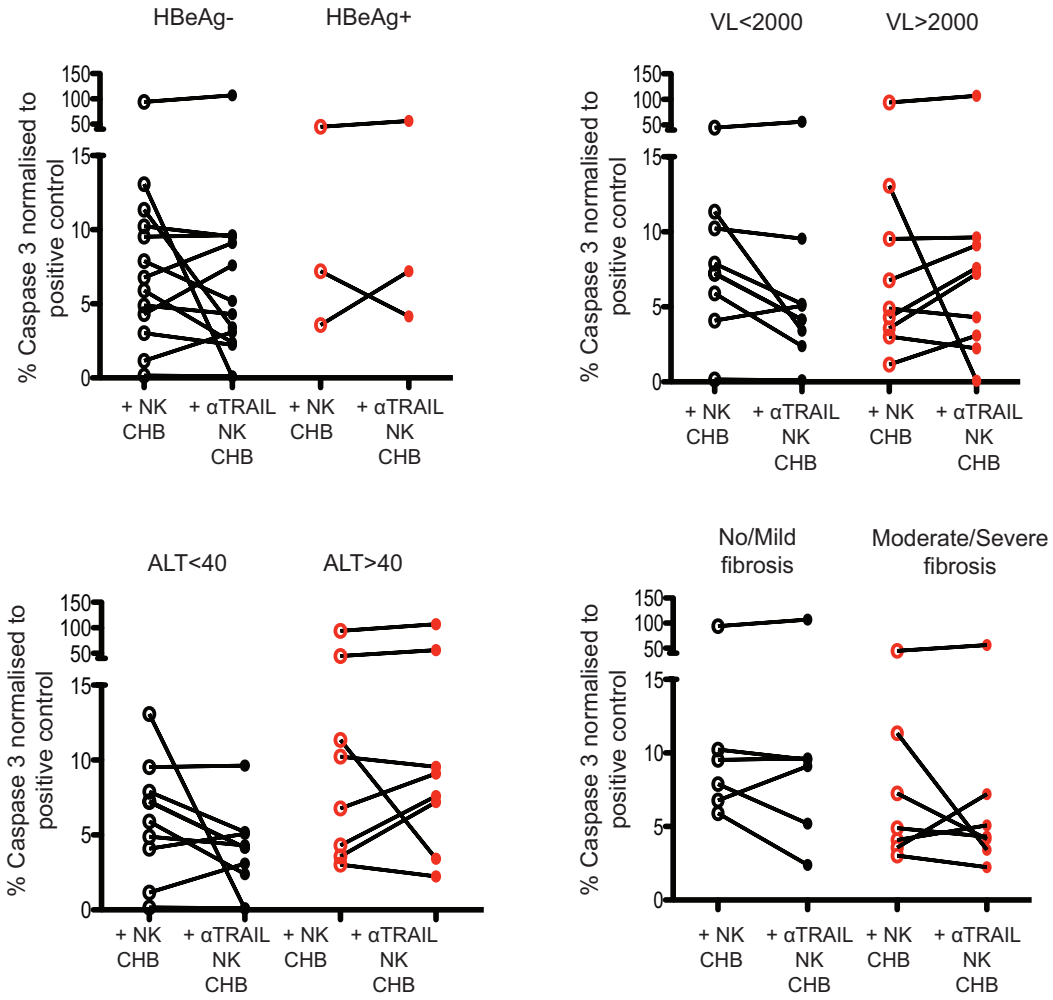


Figure 4-18: Stratification of CHB patients and the capacity of their NK cells to kill HSC post TRAIL blockade.

TRAIL was blocked on NK cells from CHB patients using neutralising antibodies against TRAIL (1μg/ml) prior to co-culture with primary human HSC. Above data depicts the potential of these NK cells to induce apoptosis in primary HSC normalized to positive control. No statistically significant difference was found in the capacity of untreated NK cells from CHB patients and TRAIL neutralised NK cells from the same CHB patients; HBeAg negative (p=0.5879, Wilcoxon paired test), HBeAg positive (p=0.5000, Wilcoxon paired test), low viral load (VL) (<2000IU/ml) (p=0.2500, Wilcoxon paired test), high viral load (>2000IU/ml) (p=0.3008, Wilcoxon paired test), low ALT (<40IU/L) (p=0.1055, Wilcoxon paired test), high ALT (>40IU/L) (p=0.2500, Wilcoxon paired test), no/mild fibrosis (p=1.000, Wilcoxon paired test) and moderate/severe fibrosis (p=0.9375, Wilcoxon paired test) to kill HSC.

Furthermore, on analysis of expression levels of TRAIL on NK cells with their capacity to induce apoptosis in HSCs, it was found that there was no correlation between these two factors as demonstrated by the correlation graph in Figure 4.19. This suggested that even though TRAIL-TRAIL receptor interaction can impact on the apoptosis of HSC, this is not solely dependent on levels of TRAIL on NK cells. The expression of TRAIL-receptors on HSC may be influencing this interaction.

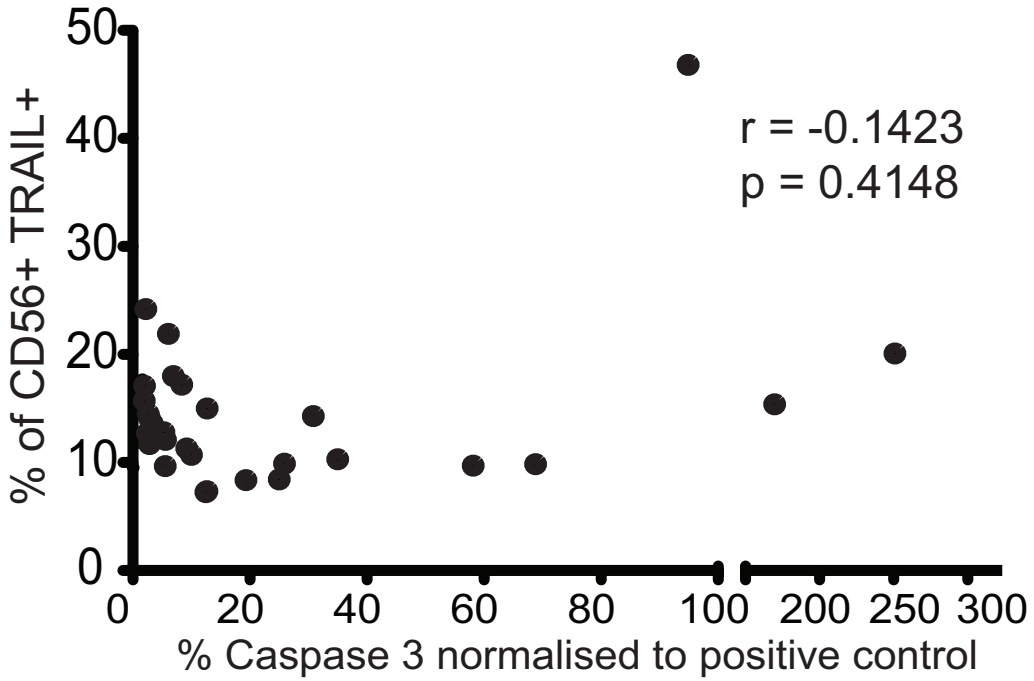


Figure 4-19: No correlation between levels of TRAIL on NK cells and their capacity to induce apoptosis of HSC.

Levels of surface expression of TRAIL on NK cells (CD56+) determined using flow cytometry (y axis) are correlated with their potential to trigger apoptosis of HSC studied as % of cleaved caspase 3 normalized to positive control (x axis). No statistical correlation is found between the two.

To further examine whether the level of TRAIL expressed on NK cells was insufficient to trigger apoptosis of HSC or whether there was a potential role of the expression of TRAIL receptors in the capacity of HSC to undergo TRAIL-mediated apoptosis, the degree of apoptosis of HSC was investigated after treating them with recombinant stabilized TRAIL ligand, *SuperKiller* TRAIL at high levels (500ng/ml). It was observed that even with such high levels of TRAIL, HSC were unable to undergo complete or high levels of apoptosis (Figure 4.20). These data imply that in this system, potentially the expression of TRAIL receptors (on the surface of HSC) may regulate or influence the capacity of HSC to undergo TRAIL-mediated apoptosis.

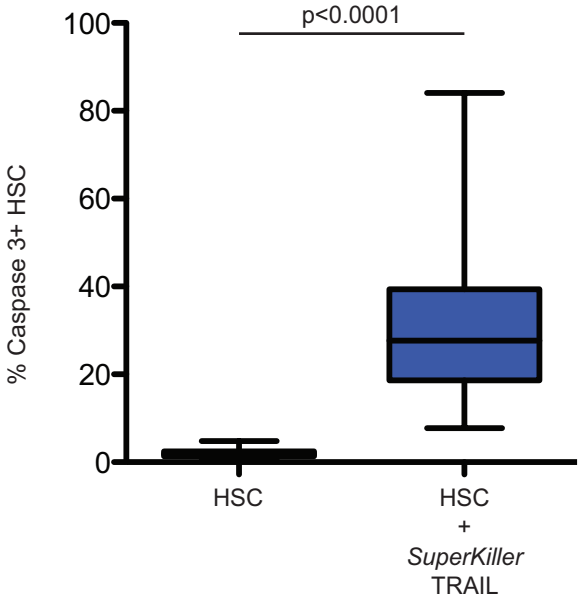


Figure 4-20: Capacity of HSC to undergo TRAIL-mediated apoptosis using recombinant TRAIL

HSC were treated with 500ng/ml of *SuperKiller* TRAIL for five hours. HSC were then stained for cleaved caspase 3 and were analysed using flow cytometry. Graph depicts (n=28) significant increase in % of expression levels of cleaved caspase 3 post treatment with *SuperKiller* TRAIL in HSC compared to untreated HSC ($p<0.0001$, Wilcoxon paired test). However, complete or high levels of apoptosis of HSC was not achieved.

Conclusion

In this chapter the *in vitro* experimental work has demonstrated that NK cells from CHB patients have a variable capacity to induce apoptosis of primary human HSC. NK cells from some healthy controls induced apoptosis of HSC, however, NK cells of CHB patients overall have a greater propensity to kill HSC. We postulate that this may be attributable to higher levels of activation and of death inducing ligands that can mediate caspase-3 mediated apoptosis on NK cells from patients with active HBV infection. This concurs with the published data showing higher levels of death inducing ligand TRAIL on NK cells of CHB patients compared to healthy controls (Dunn et al., 2007; Maini and Peppa, 2013; Rehermann, 2013).

It was observed that the NK cells of CHB patients had a variable capacity to kill HSC. For this, the patients were stratified based upon their disease status and it was observed that patients with high ALT showed a trend towards improved killing potential of HSC. This could possibly be due to increased activation and higher levels of TRAIL on the surface of NK cells of these patients (Dunn et al., 2007). It was also observed that NK cells from CHB patients with moderate to sever fibrosis had a trend towards better killing potential of HSC. There are two possible explanations for this paradoxical finding: firstly, that fibrosis progresses in these patients despite the anti-fibrogenic activity of NK cells, which may nevertheless be limiting it to some degree. Secondly, HSC of these patients may escape NK mediated death in vivo by mechanisms not fully captured by the in vitro assays.

CHB patients were also segregated on the basis of their gender and it was found that there was no statistical difference between NK cells of female or male CHB patients and their capacity to trigger apoptosis of primary human HSC. Next, we wanted to examine whether the age of the patients influenced the capacity of their NK cells to kill. No correlation was found between the age of the patients and their potential to kill HSC suggesting that age of CHB patients does not alter the function of their NK cells within the range of ages tested. However, it would be interesting to investigate whether the duration of HBV infection has an effect on the function of NK cells of patients. This data is not available for this cohort at the moment.

As previous studies have described the activation of NK cells when subjected to IFN-α, this study was taken further by activating NK cells of CHB patients and healthy individuals with rIFN-α to examine if activated NK cells have an enhanced ability to kill HSC. It was observed that irrespective of the donor status (CHB or healthy), rIFN-α activated NK cells were able to kill HSC better. Phenotyping the NK cells with and without activation with rIFN-α revealed that TRAIL was the only ligand being upregulated with this cytokine, suggesting that this enhanced capacity of NK cells to kill HSC may be mediated via the TRAIL death-inducing pathway.

To investigate whether these findings of NK cells isolated from PBMCs of CHB patients translate to the functionality of intrahepatic NK cells, preliminary experiments were carried out by isolating intrahepatic lymphocytes (IHL) from surplus tissue of liver biopsy specimens from CHB patients and IHL obtained

from liver perfusates of healthy liver donors. The n number for these preliminary experiments is very low to deduce any conclusion. However, this would be interesting to study in detail in the future as intrahepatic NK cells have a higher expression of TRAIL on their surface compared to NK cells in the periphery (Dunn et al., 2007). Also, recent publications have described a separate lineage of resident liver NK cells in mice and it would be important to study their role in this context (Daussy et al., 2014; Peng et al., 2013).

In this study, even though NK cells were able to trigger apoptosis of HSC which was further augmented by pre-treatment with rIFN-α; complete apoptosis of HSC was never attained, even with very high *in* vitro levels of TRAIL with the positive control (*SuperKiller* TRAIL).

In addition, although *in vitro* upregulation of TRAIL on NK cells did improve their capacity to kill HSC, blocking TRAIL on NK cells had no overall effect on their killing potential.

These findings prompted the question: do HSC have an anti-apoptotic mechanism switched on that limits their capacity to undergo TRAIL-mediated apoptosis?

In the next chapter we therefore examined the potential anti-apoptotic phenotype of HSC.

5. Investigating the role of TRAIL receptors in regulating apoptosis susceptibility of stellate cells

Background

Upon activation, the HSC becomes phenotypically myofibroblastic as a result of changes at gene expression level. Activated HSCs have a pro-survival phenotype that is associated with production of survival factors such as TGFβ, IGF-1, activation of NFκB and long-term changes in IκB proteins and Rel-like factors (Elsharkawy et al., 2005; Elsharkawy et al., 1999). Along with the pro-survival signals, activated HSCs have also been shown to have active anti-apoptotic signaling. An elegant study done using rodent and human HSC has shown that activated HSC overexpress Bcl-2, which makes them resistant to a variety of pro-apoptotic stimuli (Novo et al., 2006). In the same study, it was demonstrated that silencing of Bcl-2 in HSC rendered them apoptotic. However, TRAIL mediated apoptosis was not studied in this report. It is worth noting that Bcl-2 is part of the intrinsic apoptotic pathway, whereas TRAIL-induced death is a part of the extrinsic apoptotic pathway. However there are reports that the extrinsic apoptotic pathway does cross over and initiate the intrinsic apoptotic pathway and therefore can also be regulated in this manner (Falschlehner et al., 2007; Gogvadze and Orrenius, 2006). These pathways have been described in more detail in the Introduction.

Based on previous murine studies, the TRAIL mediated apoptotic pathway seems to be one of the dominant routes by which NK cells kill HSC. In the

previous chapter it has been demonstrated that TRAIL was the only marker to be significantly upregulated on NK cells treated with IFN-α *in vitro,* but we were unable to demonstrate that the baseline or IFN-α enhanced killing of HSC was TRAIL-dependent. This prompted us to examine the expression of receptors of TRAIL on HSCs to investigate the regulation of apoptosis through this pathway. We hypothesized blocking TRAIL may not reproducibly block apoptosis because it would prevent engagement of this ligand with both death-inducing and inhibitory receptors.

The death ligand TRAIL has been previously described to bind to TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 and OPG (Walczak, 2013). TRAIL can induce apoptosis by binding to TRAIL-R1 and TRAIL-R2 on target cells. TRAIL-R1 and 2 both have an intracellular death domain (DD), which is needed for downstream apoptosis-inducing signaling. TRAIL-R1 and -R2 were both discovered in 1997 and they share 58% sequence homology (Pan et al., 1997a; Pan et al., 1997b; Screaton et al., 1997; Walczak et al., 1997).

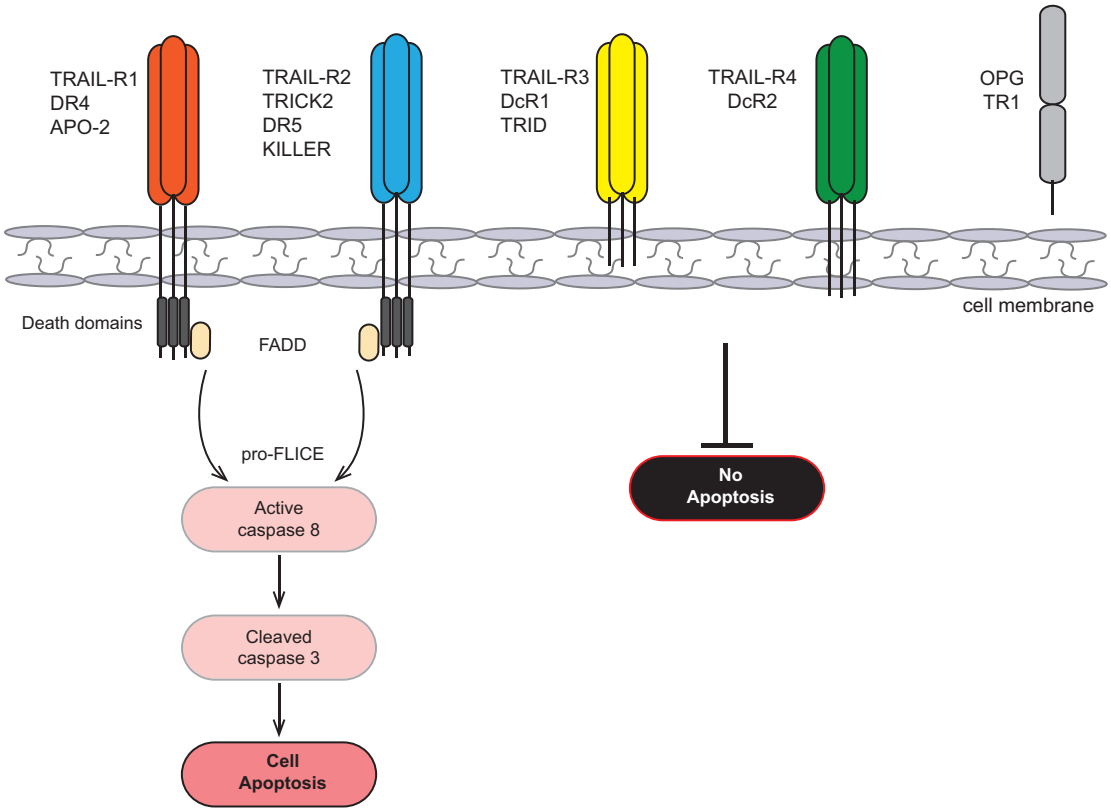


Figure 5-1: Schematic role of TRAIL receptors.

The cartoon illustrates the structures of the different TRAIL receptors and their downstream signaling. TRAIL-R1 and -R2 have transmembrane death domains; on binding to TRAIL, TRAIL-R1 and -R2 initiate downstream caspase cascade to cleave caspase 3 and induce apoptosis. TRAIL-R3 completely lacks the transmembrane death domain and TRAIL-R4 has a truncated transmembrane death domain.

However, if TRAIL binds to TRAIL-R3 or -R4 there is no downstream signaling leading to caspase-mediated apoptosis as these two receptors lack the intracellular death domain (Lalaoui et al., 2011). TRAIL-R3 and -R4 share high homology of their extracellular domain with TRAIL-R1 and -R2 (Falschlehner et al., 2007). TRAIL-R3 is held on to the membrane via its glycosyl-phosphatidylinositol tail (GPI) whereas TRAIL-R4 is anchored on to the surface of the cell through a transmembrane domain that includes a truncated DD that is unable to recruit the adaptor protein FADD (Lalaoui et al., 2011;

Meng et al., 2000). Most studies demonstrating the apoptosis preventing function of TRAIL-R3 have done so by over-expressing TRAIL-R3 on TRAIL-sensitive cells, however, their decoy role in a physiological setting still remains to be established (Falschlehner et al., 2007; Merino et al., 2006; Toscano et al., 2008). Like TRAIL-R3, TRAIL-R4 is unable to induce apoptosis, however, unlike TRAIL-R3, TRAIL-R4 has a cytosolic death domain which is truncated. Similar to TRAIL-R3, most of the studies showing the apoptosis-inhibitory role of TRAIL-R4 have been carried out in overexpression systems.

Another molecule that can inhibit downstream TRAIL-mediated apoptosis is cellular FLICE-like inhibitory protein (c-FLIP). c-FLIP is an anti-apoptotic protein that can be recruited at the site of DISC and regulate the activation of caspase-8. cFLIP is known to exist in three isoforms dependent on their amino acid lengths: $cFLIP_L$, $cFLIP_R$ and $cFLIP_S$ (Safa, 2012). cFLIP can be transcriptionally activated by changes in the microenvironment such as growth factors, cytokines, interleukins and chemotherapeutic agents (Safa et al., 2008). This molecule has homeostatic functions in normal tissue and has been reported to play a crucial role in T cell proliferation and embryonic development of certain organs (Yeh et al., 2000; Zhang et al., 2008). However, abnormal expression of this anti-apoptotic protein has been found in several diseases such as rheumatoid arthritis, cancer, Alzheimer's disease and multiple sclerosis (Safa, 2012). Animal model studies have demonstrated that suppressing the expression of cFLIP sensitizes cancer cells to death ligand mediated apoptosis and chemotherapy (Day et al., 2008).

Work looking at the expression of death inducing receptors on stellate cells has successfully shown that primary human HSC and LX2 (human HSC line) express high levels of apoptosis-inducing receptor TRAIL-R2 (Glassner et al., 2012; Taimr et al., 2003). However, there is only one published report of the expression of the apoptosis inhibitory receptors TRAIL-R3 and TRAIL-R4 on LX2 at mRNA level (Taimr et al., 2003). In this chapter we have investigated whether these receptors were also expressed at protein level on HSC and if they contributed to their anti-apoptotic phenotype. We have also examined if the expression of TRAIL receptors on HSC influences their interaction with NK cells, inhibiting/promoting TRAIL-mediated apoptosis.

5.1 Expression of TRAIL receptors of primary HSC

In every individual experiment, HSC were phenotyped for the expression of all the TRAIL receptors. As can be seen in Figure 5.2a and 5.2b, primary HSC express low levels of TRAIL -R1 and TRAIL-R3 at mRNA levels, however TRAIL-R1 is not translated at protein level; this is possibly due to post-translational modifications as described in tumor studies (Lin et al., 2010) whereas TRAIL-R3 is expressed at protein level. TRAIL-R2 and TRAIL-R4 are both highly expressed on primary HSC at mRNA and protein level.

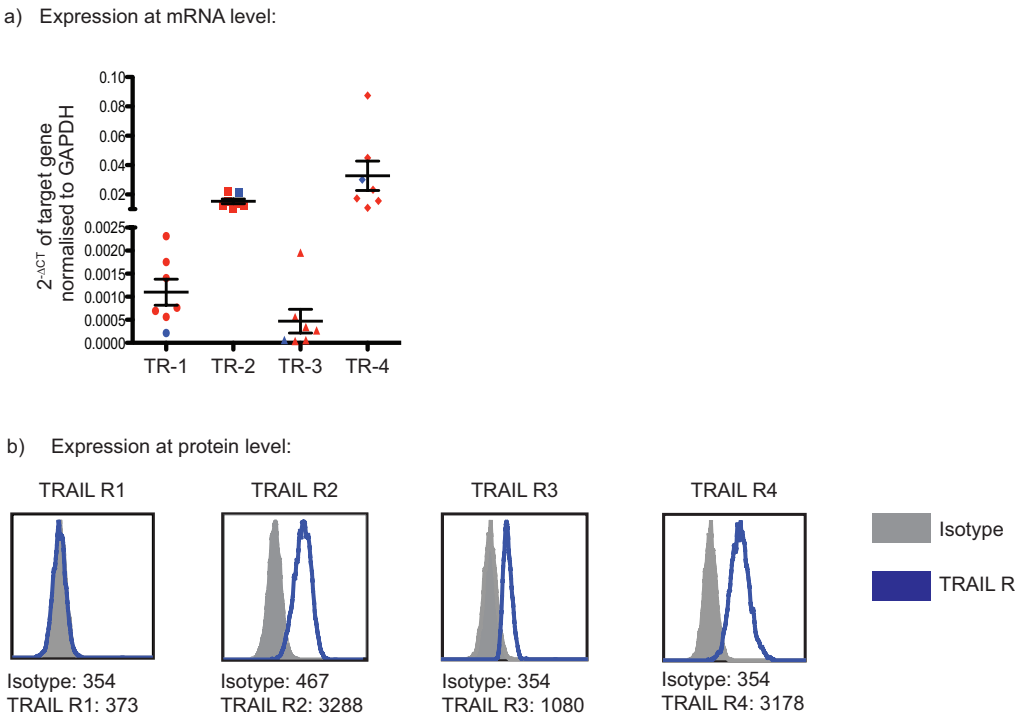


Figure 5-2: Expression of TRAIL receptors on primary human HSC.

a) mRNA levels of TRAIL-R1 (TR-1), -R2 (TR-2), -R3 (TR-3) and -R4 (TR-4) on primary human HSC from six donors (red) and LX2 (blue) b) Expression of TRAIL-R 1-4 , -R2, -R3 and -R4 at protein level using flow cytometry on primary human HSC.

5.2 Expression of TRAIL-receptors ex vivo on freshly isolated HSC

From these initial studies, it was unclear whether the pattern of TRAIL receptor expression was being modulated by *in vitro* culture of stellate cells. The expression of TRAIL-R4 and TRAIL-R3 on HSC was therefore next examined *ex vivo* on freshly isolated HSC. For this, sufficient primary HSC were obtained from six liver resections to stain these cells directly ex vivo, without the need for in vitro expansion. The gating strategy for these has been illustrated in Figure 4.3a; we excluded all other possible cell populations (Figure 4.3a legend) from the HSC fraction of the cell isolation method. To also exclude potential stem cells, CD45+ cells were gated out from HSC donor 6 and this exclusion will be included in future phenotyping. Figure 4.3b shows the expression of these receptors *ex vivo* on HSC from six different liver donors. These data reveal that HSC express apoptosis inducing receptor TRAIL-R2 and apoptosis inhibiting receptor TRAIL-R3 and TRAIL-R4 *in vivo* and this expression is not an artifact of culturing these cells. Expression levels ex vivo showed considerable variability between different donor livers as also noted after *in vitro* culture, explored further in section 5.4.

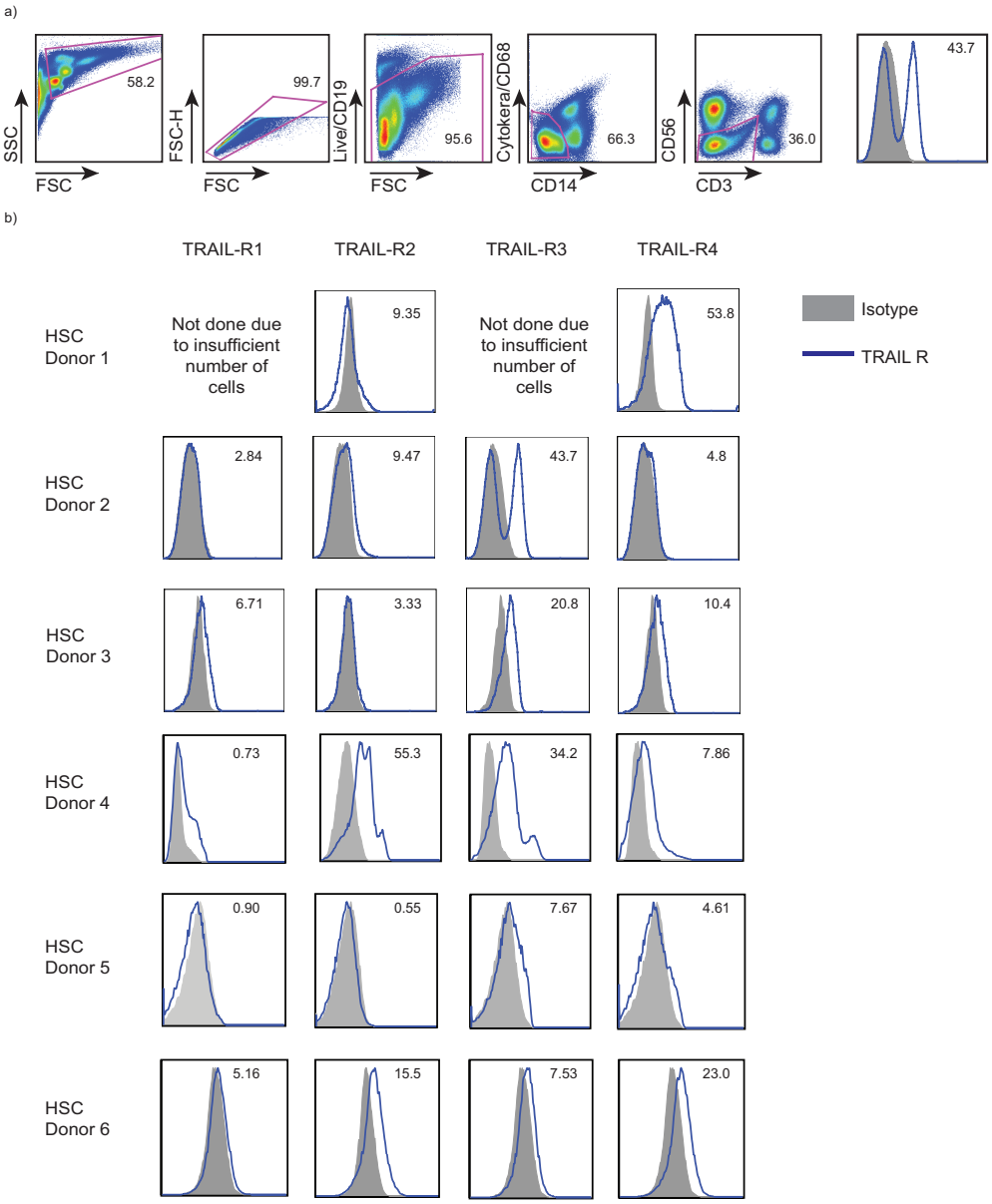


Figure 5-3: *Ex vivo* expression of TRAIL receptors on freshly isolated human HSC.

a) gating strategy of HSC *ex vivo*. Bigger cells were gated in the FSC-SSC gate, followed by excluding dead cells and B cells (CD19+); in the next gate monocytic lineages, macrophages, KC and LSEC were excluded by gating out CD14+, Cytokeratin+, CD68+ cells, CD146; next T cells, Tregs, NK cells, NKT cells and MAIT cells were excluded by gating out CD3+ and CD56+ cells; by excluding other cells we are left with HSC b) histograms showing % expression of TRAIL receptors on freshly isolated primary HSC from five different donors (blue) against matched isotype control (grey).

5.3 Functionality of inhibitory/regulatory receptor TRAIL-R3

TRAIL-R3 is an apoptosis inhibitory receptor for TRAIL. This receptor completely lacks the intracellular death domain. Overexpression experiments have demonstrated that TRAIL-R3 acts as a decoy receptor by sequestering exogenous TRAIL in the lipid rafts of target cells and via this mechanism they plausibly prevent the formation of DISC (Merino et al., 2006). To determine whether the expression of TRAIL-R3 on primary human HSC influenced their ability to undergo TRAIL mediated apoptosis, functional experiments were performed by blocking TRAIL-R3 on primary HSC and examining whether this affected their ability to undergo TRAIL mediated apoptosis. For this, primary HSC were treated with neutralising antibodies against TRAIL-R3 before the addition of *SuperKiller* TRAIL for five hours; the levels of cleaved caspase 3 were then stained and acquired using flow cytometry. As can be seen in the Figure 5.4 below, blocking TRAIL-R3 on primary HSC did improve their capacity to undergo TRAIL mediated apoptosis.

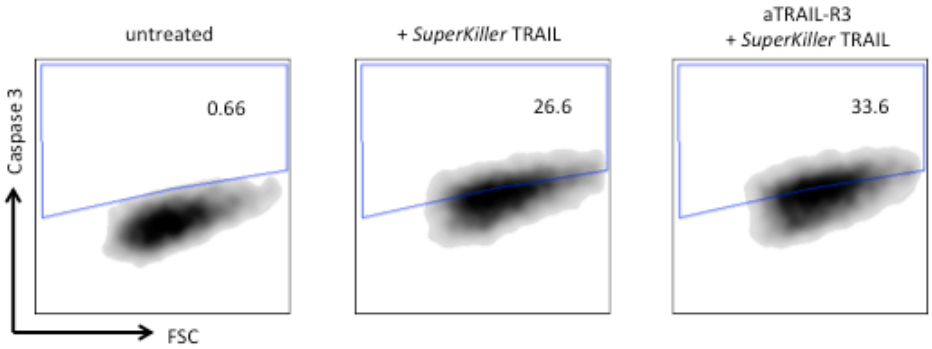


Figure 5-4: Effect of TRAIL-R3 blockade on primary HSC and their susceptibility to apoptosis.

FACS plots showing cleaved caspase 3 levels of primary HSC untreated, treated with *SuperKiller*TRAIL (500ng/ml) and treated with *SuperKiller* TRAIL after blocking TRAIL-R3 using blocking antibody (5µg/ml) on HSC.

This is the first demonstration of a decoy role of TRAIL-R3 in primary human cells. However, it is noteworthy that these experiments need to be performed on HSC from more donors and determine if this is a consistent reproducible effect.

5.4 Functionality of inhibitory/regulatory receptor TRAIL-R4

Expression of high levels of TRAIL-R4 on HSC was particularly interesting because unlike inhibitory receptor TRAIL-R3 that lacks an intracellular death domain, TRAIL-R4 has a truncated trans-membrane intra-cellular death domain. Studies done by overexpressing this receptor have reported that TRAIL-R4 can activate NFκB which can further upregulate anti-apoptotic genes and might actively contribute to protecting TRAIL-sensitive cells from apoptosis (Degli-Esposti et al., 1997). Other studies have also demonstrated that TRAIL-R4 can interact with TRAIL-R2 in a ligand dependent and independent manner and inhibit downstream caspase signaling via TRAIL-R2 (Clancy et al., 2005; Merino et al., 2006). TRAIL-R4 interaction with TRAIL, along with inhibiting the caspase cascade, may also promote cell proliferation by indirectly activating the PI3/Akt pathway (Lalaoui et al., 2011). To investigate whether the expression of TRAIL-R4 on HSC influenced their TRAIL mediated apoptosis potential, HSC were stained for TRAIL-R4 from five different donors and checked for their levels using flowcytometry. These HSC were treated with *Superkiller* TRAIL for 5 hours and the levels of active caspase-3 induced were compared using flow cytometry. Figure 5.5a shows the expression of TRAIL-R4 on HSC from all five donors. Figure 5.5b

demonstrates the levels of caspase-3 on these cells over baseline when treated with *Superkiller* TRAIL. Figure 5.5c shows striking negative correlation between the levels of TRAIL-R4 on HSC and their potential to undergo TRAIL-mediated apoptosis, implying that the higher the level of TRAIL-R4, the lower is their susceptibility to undergo TRAIL-mediated apoptosis.

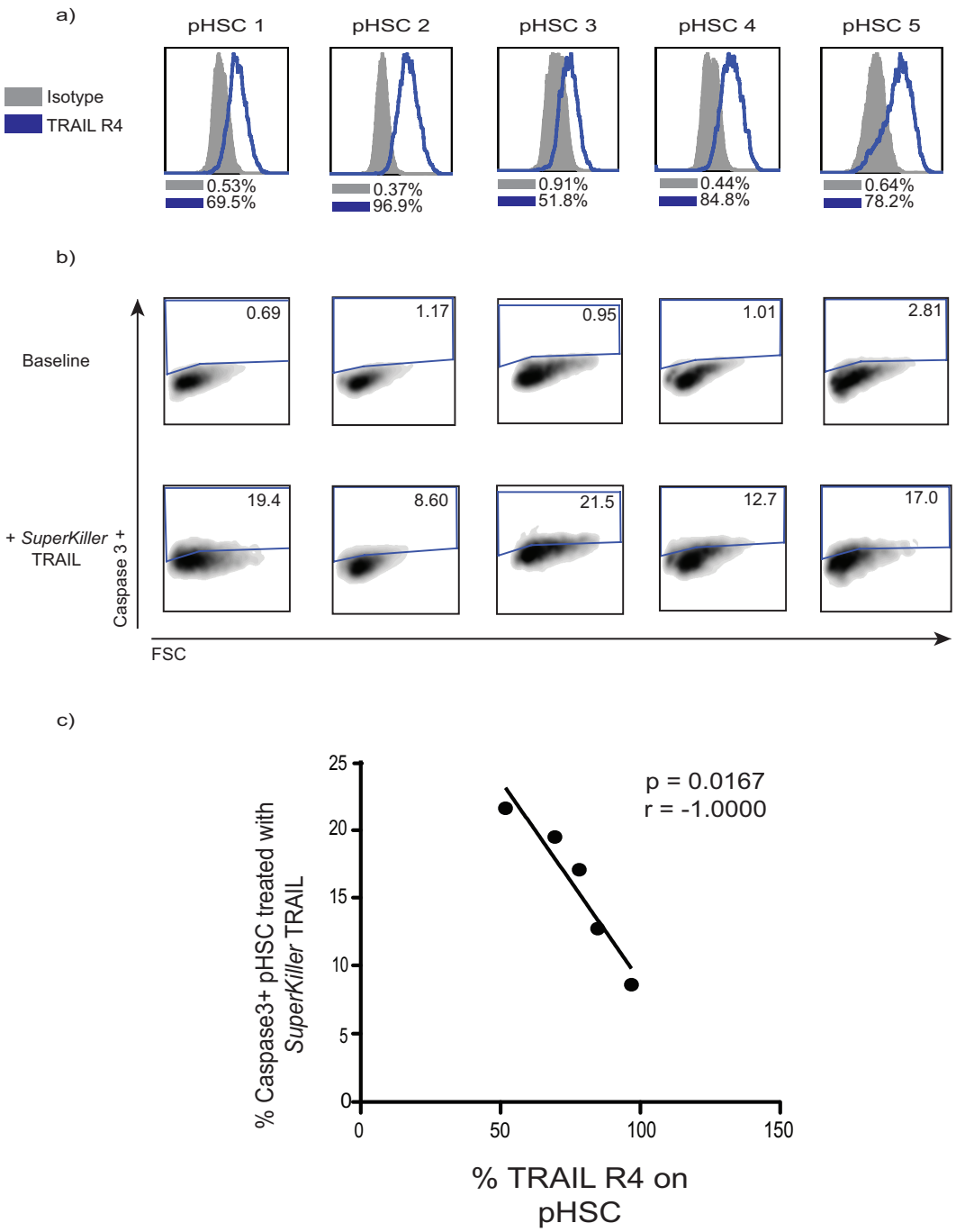


Figure 5-5: Functional effect of expression of TRAIL-R4 on HSC.

a) % Expression of TRAIL-R4 on HSC from 5 different donors using flow cytometry. b) Potential of these HSC to undergo TRAIL-mediated apoptosis. c) Correlation between expression of TRAIL-R4 on HSC and their capacity to undergo TRAIL-mediated apoptosis.

We then investigated whether the levels of TRAIL-R2 expression on primary HSC regulated the capacity of HSC to undergo TRAIL-mediated apoptosis, as was observed with TRAIL-R4 in figure 5.5. Figure 5.6 shows that no correlation was found. These data imply that even though TRAIL mediated-apoptosis of HSC occurs through TRAIL-R2, expression levels of this receptor are not the limiting factor that determines the capacity of HSC to undergo TRAIL-mediated apoptosis.

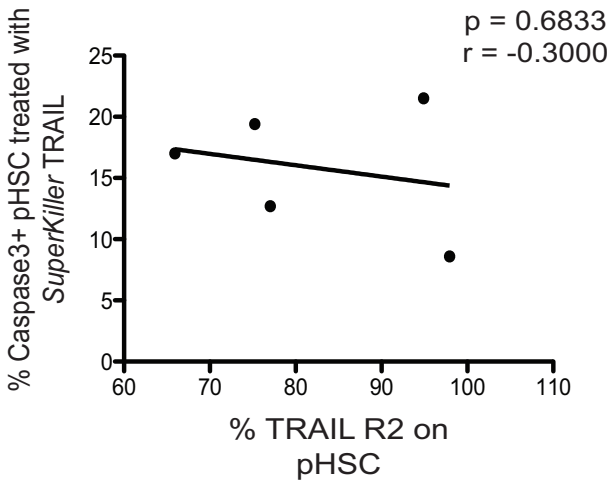


Figure 5-6: No correlation between expression levels of TRAIL-R2 on HSC and their potential to undergo apoptosis.

% expression of TRAIL-R2 on primary HSC from five donors analysed using flow cytometry (x axis) and the capacity of these HSC to undergo TRAIL mediated apoptosis (*SuperKiller* TRAIL, 500ng/ml) assessed by % of cleaved caspase 3 using flow cytometry (y axis) plotted in a correlation graph. No Correlation between expression of TRAIL-R2 on primary HSC and their capacity to undergo TRAIL-mediated apoptosis was observed..

Furthermore, the effect of blocking TRAIL-R4 was examined on these HSCs. For this, purified monoclonal antibody was used to block TRAIL-R4 on HSC and it was found that blocking TRAIL-R4 did increase the induction of apoptosis in HSC in some cases and not in others (Figure 5.7). It is noteworthy that three primary human HSC lines out five responded to TRAIL-R4 blocking, enhancing their potential to undergo TRAIL mediated apoptosis. The response to blockade did not correlate with baseline levels of TRAIL-R4 expression (Figure 5.5a) on these primary HSC.

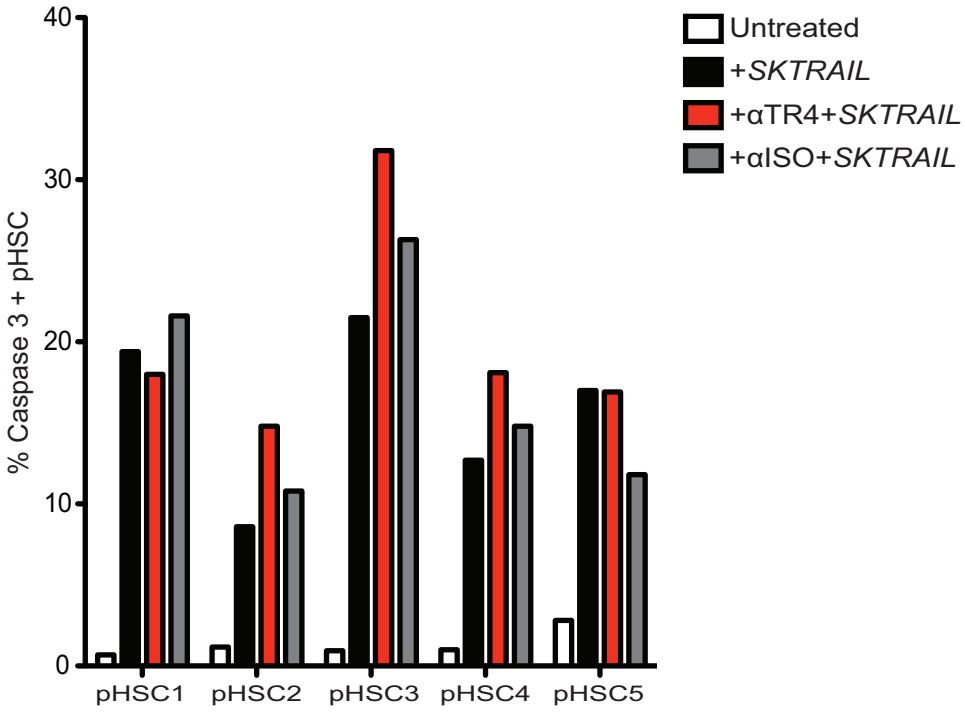


Figure 5-7: Effect of blocking TRAIL-R4 on HSC and their capacity to undergo TRAIL-mediated apoptosis

TRAIL-R4 was blocked on primary HSC using neutralising antibody (5µg/ml) before adding *SuperKiller* TRAIL (500ng/ml) to assess their capacity to undergo TRAIL mediated apoptosis. Flow cytometry analysis of % cleaved caspase 3 is used as a readout. Graph showing cleaved caspase 3 levels on untreated primary HSC, treated with *SuperKiller* TRAIL with or without pre-treatment with a monoclonal antibody to block TRAIL-R4 or an isotype control for blocking.

This once again underscored the inter-donor variability and suggested HSC from different individuals have differential dependence on the different TRAIL receptors to regulate apoptosis. This could also represent the affinity with which the blocking antibody binds to each separate primary HSC line. To overcome any non-specific binding or low affinity binding of blocking antibody against TRAIL-R4, we developed a system using short hairpin against TRAIL-R4 (shTRAIL-R4) delivered using a lentiviral vector to knock-down the expression of TRAIL-R4 in HSC. This work was done in collaboration with Itziar Otano (Postdoc, Maini group). As a proof of principle this was first optimised using LX2. As can be seen in Figure 5.8, LX2 were effectively transduced with lentivirus (GFP+). TRAIL-R4 expression was knocked-down in LX2 transduced with lentivirus containing shTRAIL-R4 compared to those transduced with lentivirus containing a control short hairpin.

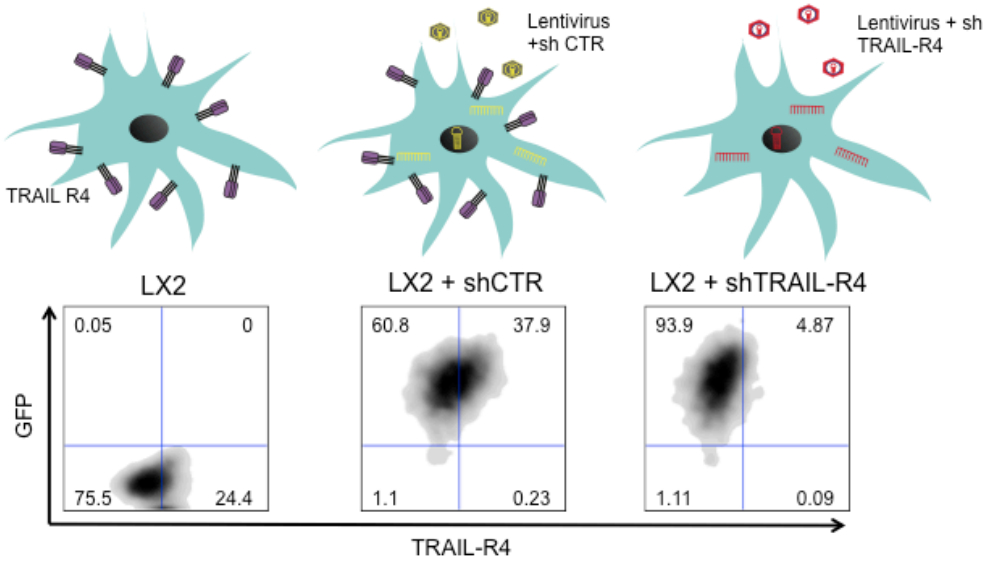


Figure 5-8: TRAIL-R4 knock-down of HSC.
Using lentivirus to deliver short hairpin against TRAIL-R4 to knock it down. Diagramatic illustration and FACS plot showing expression of TRAIL-R4 against levels of transduction (GFP+) in untransduced LX2 or LX2 transduced with short hairpins against TRAIL-R4 or control.

Next, to examine whether knock-down of TRAIL-R4 on the surface of LX2 made them more susceptible to TRAIL mediated killing, untransduced LX2, LX2 transduced with control lentivirus (ctrLV), LX2 transduced with lentivirus containing shTRAIL-R4 (shTR4) and LX2 with TRAIl-R4 neutralised using blocking antibody (aTR4) were treated with *SuperKiller* TRAIL (500 ng/ml) for five hours and their levels of cleaved caspase 3 were analysed using flow cytometry (Figure 5.9). This experiment was done with three technical replicates for each condition. It was observed that LX2 transduced with shTRAIL-R4 and LX2 treated with blocking antibody against TRAIL-R4 both had an improved potential to undergo TRAIL-mediated apoptosis compared to apoptosis in untransduced LX2 and LX2 transduced with control lentivirus.

This is however, a preliminary experiment done on LX2, we would like to repeat this in primary human HSC from different donors. It is noteworthy that unlike primary HSC, LX2 do not express TRAIL-R3, therefore antibody blockade or knocking down TRAIL-R3 using lentivirus was not possible in this cell line.

It is worth noting that there was considerable amount of caspase 3+ LX2 at baseline with TRAIL-R4 blocking using neutralising antibody. These data could suggest that the TRAIL-R4 monoclonal antibody could be partially stimulatory or that HSC are dependent on tonic survival signals from TRAIL-R4 even in absence of ligand.

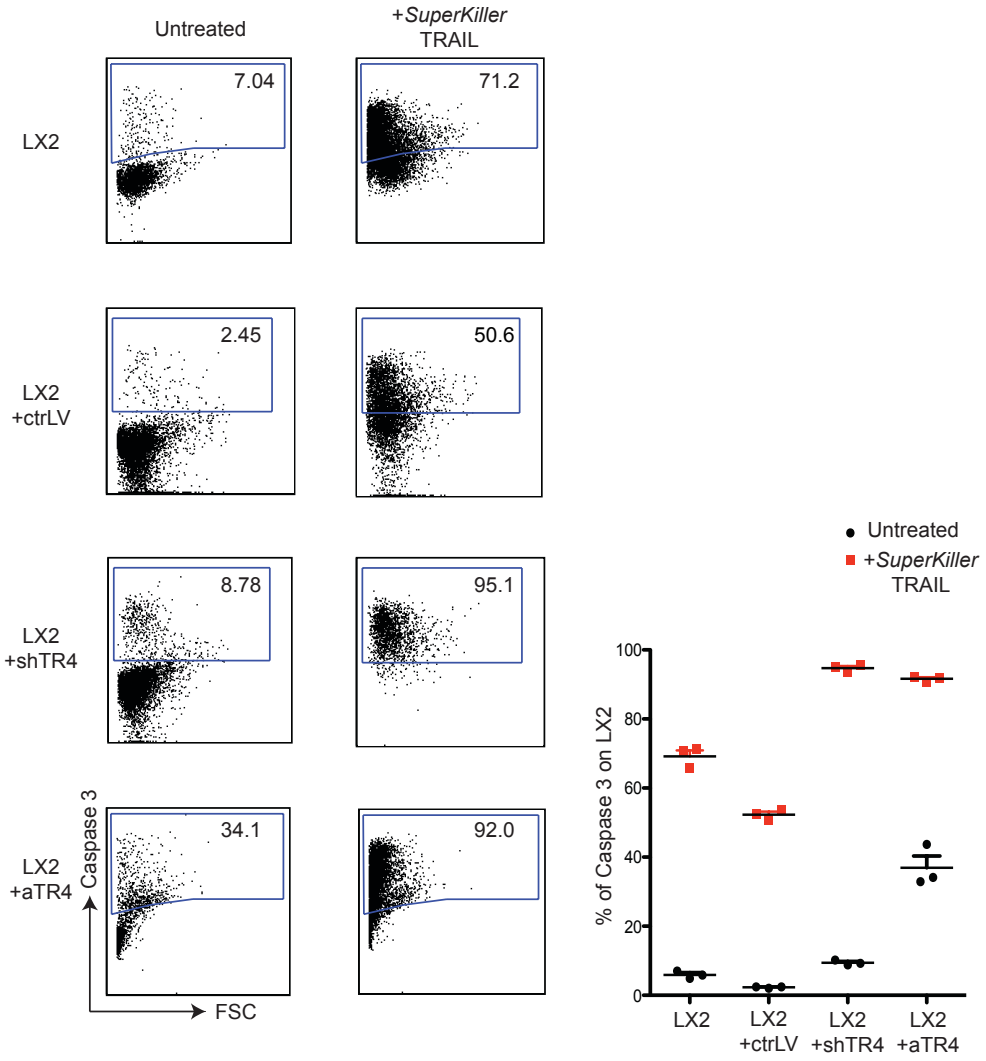


Figure 5-9: Using lentiviral vectors to knock-down expression of TRAIL-R4 on HSC and their susceptibility to undergo TRAIL mediated apoptosis.

Representative FACS plots showing % expression of cleaved caspase 3 on untreated and *Superkiller* TRAIL (500ng/ml) treated untransduced LX2, LX2 transduced with control lentivirus (ctrLV), LX2 transduced with lentivirus containing shTRAIL-R4 (shTR4) and LX2 neutralised with TRAIL-R4 using blocking antibody (aTR4) (5µg/ml). Cumulative graph showing technical replicates of this experiment.

Having demonstrated that expression of TRAIL-R3 and TRAIL-R4 could modulate the susceptibility of primary HSC to apoptosis induction by an artificial form of TRAIL-ligand, our next aim was to investigate if this was also the case for NK cells endogenously expressing TRAIL.

5.5 Functional effect of expression of TRAIL-R3 and -R4 on HSC on their susceptibility to NK cell mediated death

To investigate this, TRAIL-R3, TRAIL-R4 or both were neutralised on HSC, which were then co-cultured with NK cells with or without *in vitro* recombinant IFN-α treatment (to upregulate their TRAIL expression). Figure 5.10a illustrates the experimental design of this assay. As can be seen in Figure 4.10b, blocking TRAIL-R4 on primary HSC using neutralising antibody significantly increased the susceptibility of HSC to undergo TRAIL-mediated apoptosis by NK cells. On some of the patients from Figure 5.10b, TRAIL-R3 was also neutralized on primary HSC. Data represented in Figure 5.10c depicts the improved susceptibility of primary HSC to undergo apoptosis upon co-culture with NK cells from CHB patients after blocking TRAIL-R3 and TRAIL-R3+TRAIL-R4 on the surface of primary HSC.

Figure 5.11 shows the caspase 3 levels of primary HSC upon co-culture with NK cells from individual CHB patients and the levels of caspase 3 after blocking TRAIL-R3 or TRAIL-R4 or both on primary HSC. The data shows that blocking TRAIL-R3,-R4 or both does make HSC more susceptible to NK cell mediated killing in some cases while it has no effect in other cases. These

differences are consistent with human data examining *in vitro* blockade of molecules such as PD-1, CTLA-4 etc. owing to the heterogeneity in patient cells.

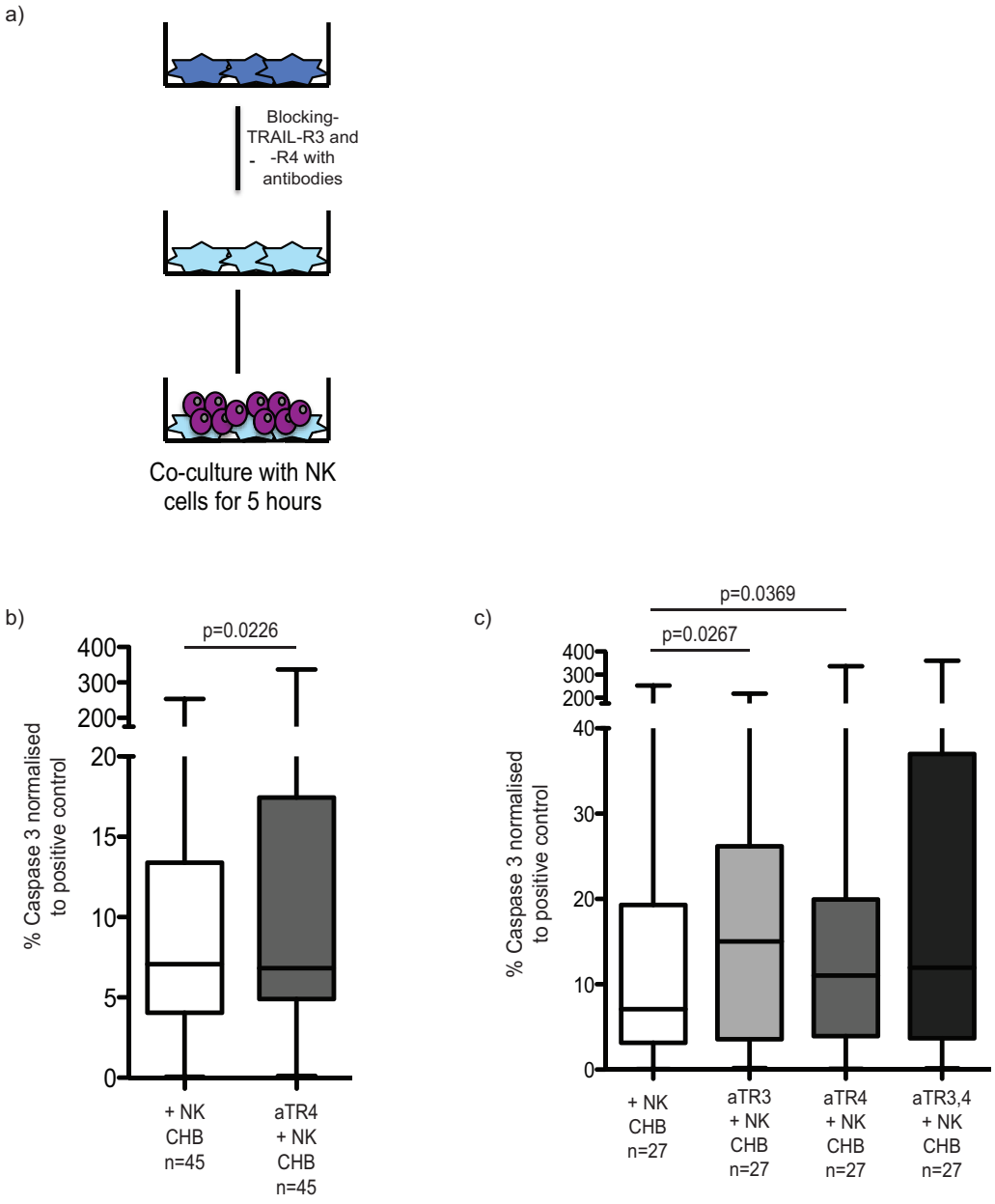


Figure 5-10: Effect of NK-HSC co-culture post neutralising TRAIL-R3 and -R4 and combination on HSC.

NK cells were co-cultured with HSC after TRAIL-R3 and TRAIL-R4 surface expression was blocked on HSC using neutraling antibodies (5µg/ml); their capacity to undergo apoptosis was determined by staining for caspase 3 and analysed using flow cytometry. Graphs represent levels of caspase 3 normalised to positive control (*SuperKiller* TRAIL). a) diagrammatic representation of experimental design. b) Significant difference was found in the capacity of HSC to undergo NK-mediated apoptosis after blocking TRAIL-R4 (0.0226, Wilcoxon-paired test) c) significant difference was found in apoptosis of HSC upon blocking TRAIL-R3 and combination blockade of TRAIL-R3 and –R4 and co-culture with NK cells (0.0153, Friedman test one-way ANOVA).

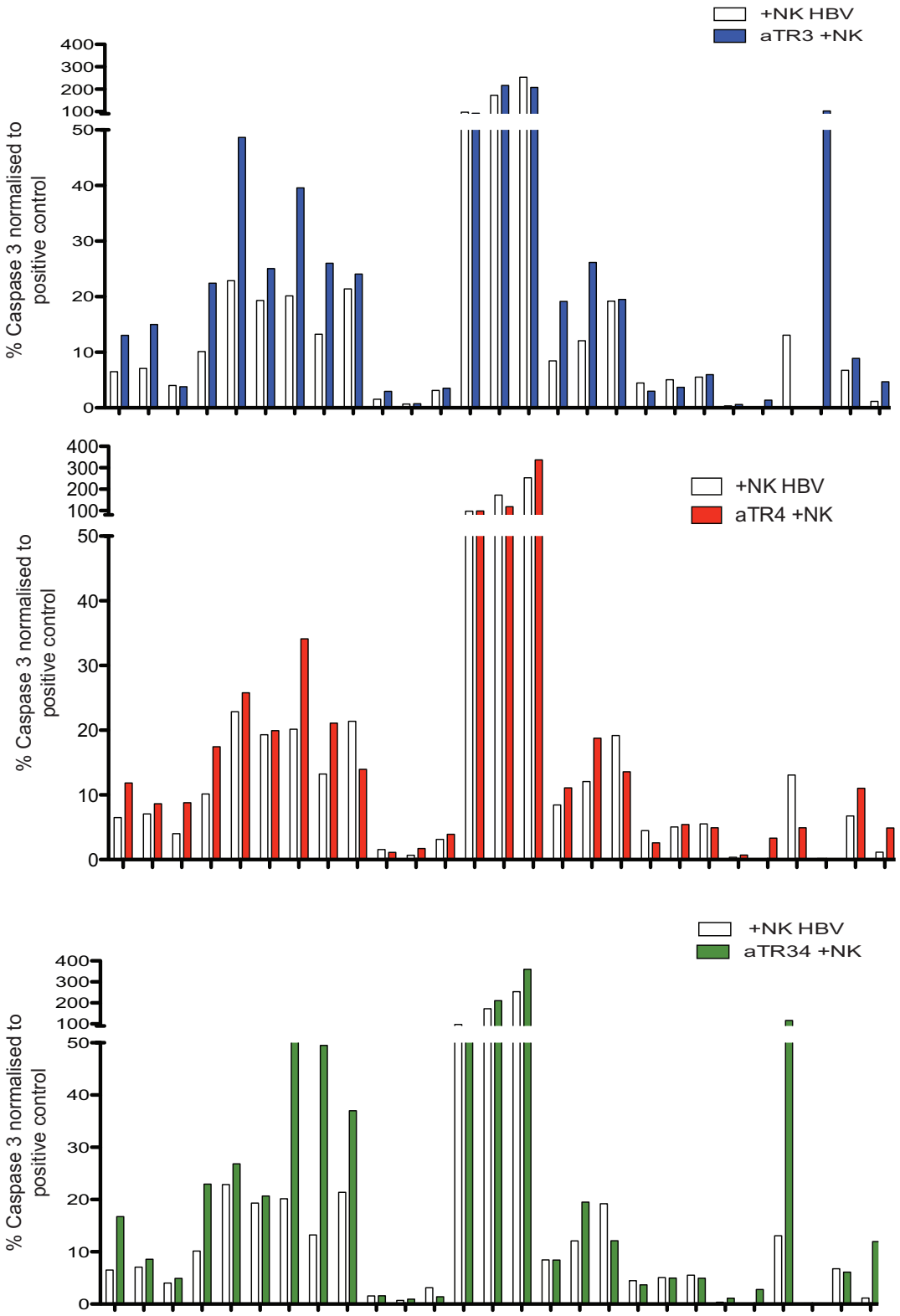


Figure 5-11: Individual patient data for effect of NK-HSC co-culture post neutralising TRAIL-R3 and -R4 and combination on HSC.

NK cells were co-cultured with HSC after TRAIL-R3 and TRAIL-R4 surface expression was blocked on HSC using neutralising antibodies (5μg/ml); their capacity to undergo apoptosis was determined by staining for caspase 3 and analysed using flow cytometry. Graphs represent levels of caspase 3 normalised to positive control (*SuperKiller* TRAIL). White bars represent apoptosis of primary human HSC post co-culture with each CHB patients. Blue, red and green bars show apoptosis of primary human HSC after TRAIL-R3 blockade, TRAIL-R4 blockade, TRAIL-R3,4 blockade using respective neutralising antibodies post co-culture with each CHB patients.

Next, experiments were done to examine whether HSC, when their inhibitory TRAIL receptors were blocked, had an increase in susceptibility to undergo apoptosis when co-cultured with NK cells that were pre-activated using rIFN-α (to upregulate their levels of TRAIL expression). As can be seen in Figure 5.12b, blocking TRAIL-R4 on HSC showed a significant increase in their susceptibility to TRAIL-mediated killing by NK cells activated with IFN-α compared to untreated NK cells. It was found that (Figure 5.12a, c) blocking TRAIL-R3, the combination of TRAIL-R3 and-R4 on HSC showed a non-significant trend to make them more susceptible to TRAIL-mediated killing by NK cells activated with IFN-α compared to untreated NK cells from CHB patients.

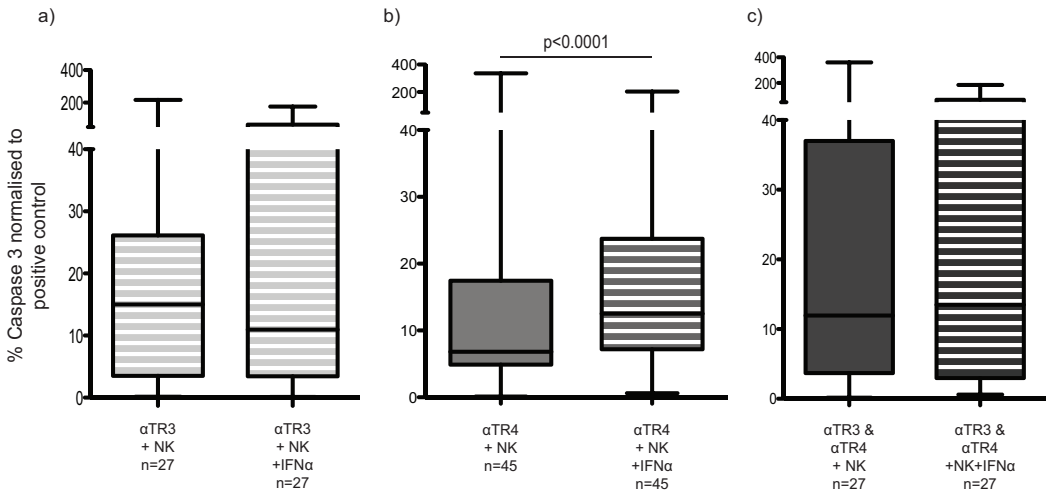


Figure 5-12: Effect of NK (untreated and rIFN-α treated)-HSC co-culture post neutralising TRAIL-R3 and -R4 and combination on HSC.

NK cells were co-cultured with HSC after TRAIL-R3 and TRAIL-R4 surface expression was blocked on HSC using neutralising antibodies (5μg/ml); their capacity to undergo apoptosis was determined by staining for caspase 3 and analysed using flow cytometry. Graphs represent levels of caspase 3 normalised to positive control (*SuperKiller* TRAIL). a) NK cells of CHB patients treated with rIFN-α *in vitro* have a non-significant trend of improved capacity to kill primary HSC (post neutralising TRAIL-R3) (p=0.3426, Wilcoxon paired test). b) NK cells of CHB patients treated with rIFN-α *in vitro* have a significant improved capacity to kill primary HSC (post neutralising TRAIL-R4) (p<0.0001, Wilcoxon paired test). c) NK cells of CHB patients treated with rIFN-α *in vitro* have a non-significant trend of improved capacity to kill primary HSC (post neutralising TRAIL-R3 and –R4) (p=0.6159, Wilcoxon paired test)

These data indicate that upon upregulating TRAIL levels on NK cells after pre-treating them with IFN-α and co-culturing them with HSC that have TRAIL-R3, -R4 or the combination blockade, NK cells did show a significant improvement in their potential to kill HSC. It is noteworthy that in some cases blocking TRAIL-R3 and/or -R4 greatly improved the killing potential of activated NK cells while in other cases blocking these receptors did not affect the killing potential of NK cells. It is worth noting that the primary HSC lines used for these experiments were the ones that responded to TRAIL-R4 blockade from Figure 5.7 (pHSC2,3,4). It is also worth noting that unlike LX2 (figure 5.9), primary HSC did not increase their baseline apoptosis on treatment with TRAIL-R4 neutralising antibody, this could suggest that in LX2, blocking TRAIL-R4 could have a ligand independent effect that drives their apoptosis and is enhanced on ligand engagement.

5.6 Other potential mechanisms that may suppress TRAIL mediated apoptosis of HSC

As described in the Introduction section of this thesis, apoptosis is a very organized and regulated mechanism of programmed cellular death that can occur in two ways: via the intrinsic pathway, also known as the mitochondrion-initiated pathway; and via the extrinsic or cell surface death receptor pathway (Elmore, 2007; Gogvadze and Orrenius, 2006). TRAIL – TRAIL receptor interactions fall under the extrinsic apoptotic pathway. In this pathway, other than interaction with TRAIL-R3 and TRAIL-R4, there is another downstream molecule that can inhibit apoptosis referred to as the cellular FLICE-like inhibitory protein (c-FLIP). c-FLIP is an anti-apoptotic protein that can be recruited at the site of DISC and regulate the activation of caspase-8 as illustrated in the Figure 5.13 below.

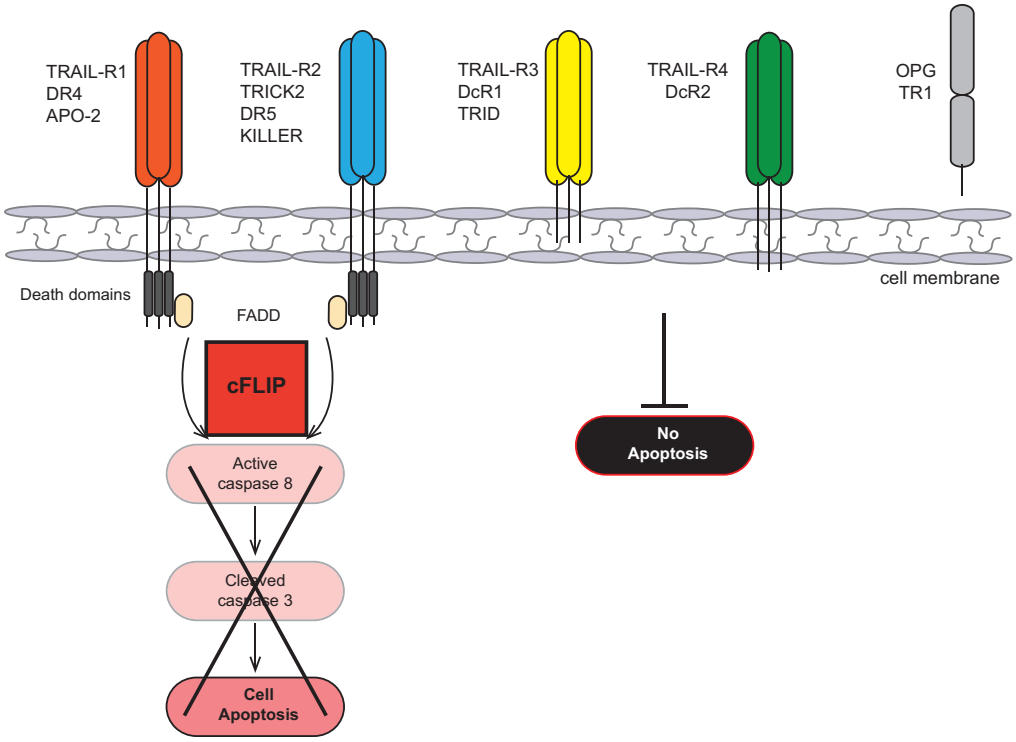


Figure 5-13: cFLIP blocks downstream TRAIL-mediated apoptosis.
Schematic diagram of cFLIP preventing downstream caspase mediated apoptosis by engaging with DISC.

Expression of cFLIP in primary human HSC was examined using flow cytometry and it was revealed that HSC express high levels of cFLIP as shown in Figure 5.14.

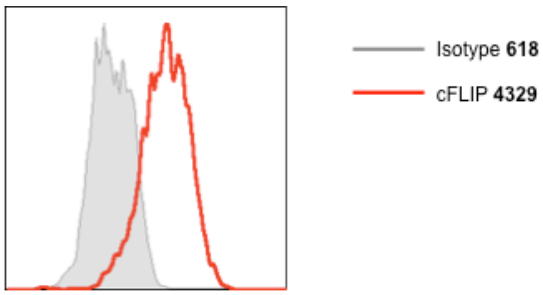


Figure 5-14: High expression of cFLIP in primary human HSC.
Histogram of FACS staining of cFLIP on primary HSC in red against matched isotype control in grey.

Expression of cFLIP is another potential mechanism to protect HSC from TRAIL mediated apoptosis and facilitate fibrosis. However, there is a need to check this finding with technical and biological (other primary HSC donors) replicates and their functional role needs to be investigated further. cFLIP could be a potential reason why no augmentation on NK cell-mediated apoptosis in seen in HSC after blocking inhibitory TRAIL-R3 and TRAIL-R4.

Along with the anti-apoptotic role of TRAIL-R3 and TRAIL-R4 as described in this thesis, high expression of cFLIP and Bcl-2 on HSC may be plausible complementary mechanisms that allow escape of HSC from TRAIL-mediated apoptosis.

Conclusion

In this chapter it has been demonstrated that primary human HSC express high levels of apoptosis-inducing TRAIL-R2 along with apoptosis-inhibitory TRAIL-R3 and regulatory TRAIL-R4.

Further investigation revealed that expression of TRAIL-R4 inversely correlated with induction of TRAIL-mediated apoptosis on HSC, implying that TRAIL-R4 plays a dominant role in regulating TRAIL-mediated apoptosis in these cells, irrespective of their expression of other TRAIL receptors. These data suggest that in patients with fibrosis, the expression of TRAIL-R4 on their activated HSC could determine the potential for resolution of fibrosis. This is the first report of a direct correlation between expression of TRAIL-R4 on a primary cell type and its potential to undergo apoptosis. However, this needs to be investigated further in *in vivo* models.

The functional role of expression of these receptors was examined by co-culturing them with NK cells from CHB patients with and without pre-activation with rIFN-α. It was observed that blocking these receptors did not significantly enhance the capacity of NK cells to kill HSC. This could possibly be due to the affinity with which the blocking antibodies bind to HSC. Another system has been setup to overcome any drawbacks of using neutralising antibody. A lentiviral system was developed to deliver short hairpin against TRAIL-R4 to knock-down its expression in HSC. Data in this chapter show successful transduction of LX2 with these and an augmentation in their capacity to undergo TRAIL mediated apoptosis. In the future, this system will be used in

primary human HSC from different donors to determine whether knock-down of TRAIL-R4 makes primary HSC as susceptible to TRAIL mediated apoptosis as LX2.

The influence of expression levels of TRAIL-receptors on their capacity to undergo TRAIL-mediated apoptosis as described in this chapter brings to light the fact that correcting for apoptosis based on TRAIL as a positive control is probably not ideal for looking at variability attributable to these receptors. In the future, we would like to re-analyse data without correcting against the positive control and instead analysing according to status of primary HSC in terms of balance of different TRAIL receptors.

The outcome and the future perspective of this study is discussed further in the Discussion section of this thesis.

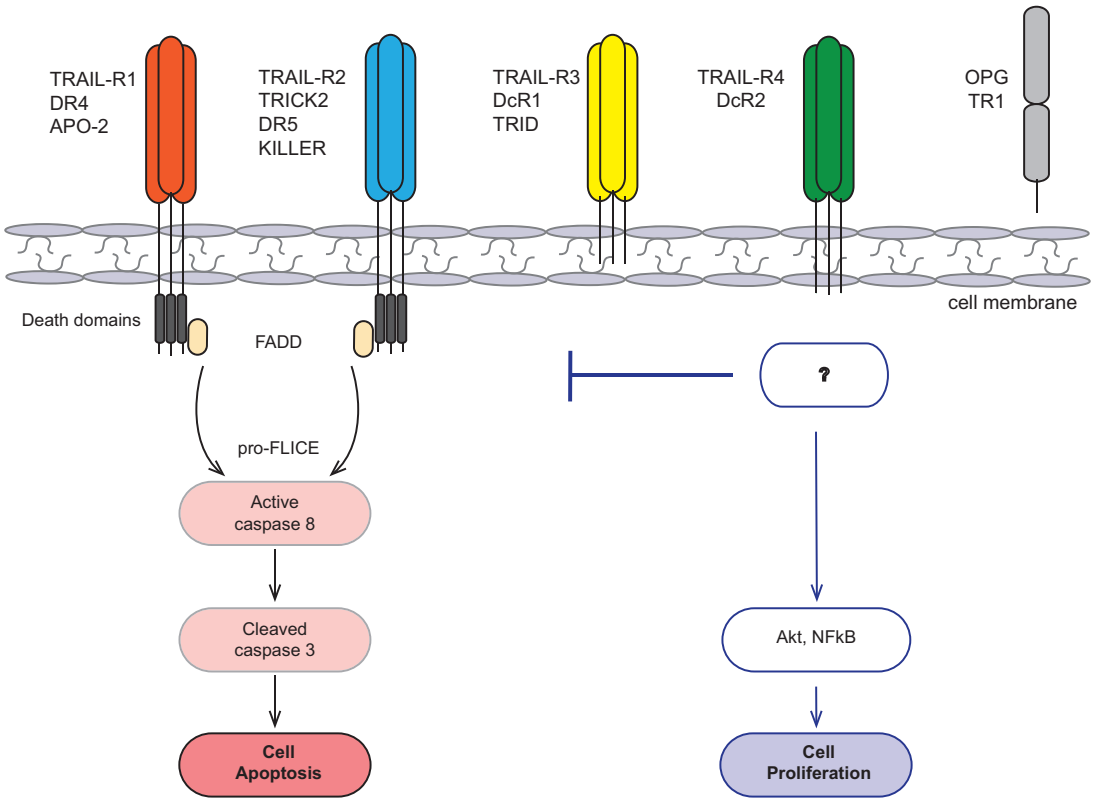


Figure 6-15: Potential downstream signaling of TRAIL-R4.

Cartoon showing the different TRAIL receptors and their downstream signaling interactions. TRAIL-R4 has a truncated transmembrane death domain. Two publications have overexpressed TRAIL-R4 on cell lines and demonstrated that TRAIL-R4 has the capacity for downstream activation of the NFκB and PI3/Akt pathways leading to cell survival and proliferation (Degli-Esposti et al., 1997; Lalaoui et al., 2011). The mechanism through which this downstream mechanism is initiated remains to be investigated.

6. Conclusion and Discussion

Summary and Discussion of work presented

Chronic infection with HBV can lead to cirrhosis and/or HCC, which are the causes of mortality in this disease. Liver cirrhosis is the end stage of fibrosis, which is the culmination of scar tissue formation over an extended period of time, as a result of persistent liver injury. In CHB, the rate at which fibrosis develops differs from patient to patient but population studies suggest that the incidence of cirrhosis in patients with CHB followed in clinics is 2-3% per year (Cabibbo et al., 2012; Di Marco et al., 1999; Fattovich et al., 1991) and the incidence of decompensated cirrhosis is 0.5 per 1000 person-years (McMahon et al., 2001). Currently, there are no drugs licensed for the treatment of liver fibrosis, though many are under investigation. Since HBV is a non-cytopathic virus, the damage caused to the liver by this infection is a result of the immune responses it triggers. In this thesis, we have examined the role of immune responses in driving HBV-related liver fibrosis.

In chapter 2 of this study, it was shown for the first time, with primary samples from patients infected with CHB, that peripheral blood mononuclear cells from these patients produce soluble mediators that can activate HSC (LX2) at an mRNA and protein level. There is a possibility of more than one cell type contributing to this observation.

Previous work in our group by Lopes *et al.* showed an upregulation of various pro-fibrogenic genes, such as, connective tissue growth factor (CTGF),

fibroblast growth factor (FGF), insulin-like growth factor binding protein 3 (IGFbp-3) (Lopes et al., 2008) in virus-specific $CD8^+$ T cells from patients with persistent compared to resolved HBV infection. ELISA were performed with supernatants from these PBMC cultures for CTGF and IGFBP-3. No expression of IGFBP-3 at protein level was found. Expression of CTGF was observed, however this was not conclusive and could not be correlated with disease status. This could be because the experimental timing may have been wrong to detect the release of these factors. The cells might need longer than 24 hours to secrete fibrotic factors. Another explanation for this might be that the impact of these fibrotic factors from HBV-specific $CD8^+$ T cells is swamped by a stronger effect from some other constituent of PBMC or that HBV-specific $CD8^+$ T cells do not produce pro-fibogenic factors detectable at protein level

Studies performed by Fowell *et al.* in the Rosenberg group, have investigated the role of PBMC from HCV infected patients in liver fibrogenesis (Fowell, 2008). It was found that on stimulation with HCV proteins, PBMC from HCV infected patients with fast progressing fibrosis are able to produce soluble factors that can activate HSC (LX2 in this case) at mRNA level (αSMA and Pro-collagen I) more than PBMC from HCV infected patients with slow progressing fibrosis and healthy controls. Since they used HCV proteins in their experimental design to stimulate the PBMC from HCV infected patients, it is possibly the $CD4^+$ T cells in the system that were responding to the protein stimulation. They observed that HCV proteins alone did not have an effect of enhancing LX2 activation and concluded that the effects observed

were mediated by a PBMC fraction but did not refine whether or not this effect was attributable to innate or adaptive immune responses.

Other groups working on regulatory T cell (Treg) have shown that in HBV infection, the balance between T helper 17 cells and Tregs is able to influence the progression of liver fibrosis by activating HSC (Li et al., 2012).

NKT cells and especially MAIT cells are additional possible candidates for the cells responsible for driving the activation of LX2 in our study. NKT cells are known to drive fibrosis directly in the liver in a CXCR6 dependent manner and by secreting pro-fibrotic cytokines in HBV transgenic mice models and also indirectly by stimulating other immune cells in the liver to produce pro-inflammatory cytokines (Gao et al., 2009; Jin et al., 2011; Swain, 2010; Wehr et al., 2013). MAIT cells have been shown to be abundant in the human liver. On stimulation they produce pro-fibrogenic cytokines such as IL17A and TNFα (Dusseaux et al., 2011; Tang et al., 2013; Walker et al., 2012).

Macrophages have an important pro-fibrogenic role (Duffield et al., 2005; Imamura et al., 2005; Meng et al., 2012; Negash et al., 2013). Studies in HCV have implicated their pro-fibrogenic role via IL1β (Negash et al., 2013; Shrivastava et al., 2013). It would be especially interesting to study the role of intra-hepatic macrophages and Kupffer cells from CHB livers in activating HSC.

Even though the PBMC from CHB patients were able to significantly activate HSC more than PBMC from healthy controls, there was a large spread of pro-fibrotic responses amongst the patient cohort. The CHB patients were stratified into various disease stages to dissect their fibrotic potential better. We compared them on their degree of fibrosis determined by Ishak stage and/or ELF scores. It is worth noting that we did not have many patients with severe or advanced fibrosis and therefore we have separated them into those with an Ishak fibrosis stage below 2 and ELF score below 7.7 as mild/no fibrosis and those with an Ishak fibrosis stage between the range of 2-4 and ELF score between the range of 7.7 – 9.8 as having moderate fibrosis. We did not see any differences in the potential of their PBMC to activate HSC. We need to test the fibrotic potential of PBMC of CHB patients with severe fibrosis or cirrhosis to study this stratification better.

The viral load and HBeAg status (data not shown) of the patients were also compared and these comparisons did not show any differences in pro-fibrotic potential of PBMC according to viral load and HBeAg status.

However, in this study it was observed that patients with increasing liver inflammation, measured by ALT, show a trend towards greater stellate cell activation. This was seen consistently, both at mRNA and protein levels with all the four markers of activation. This could possibly be because, during inflammation the immune cells are activated further. This could result in them producing more pro-fibrogenic soluble mediators to activate stellate cells to initiate the wound-healing mechanism. Our preliminary findings showing more

association with ALT than fibrosis score, raises the possibility that the PBMC pro-fibrogenic effect may be more relevant in initiating fibrosis than in perpetuating it once it is established. Further investigation of this would require longitudinal follow-up studies with much larger cohorts of patients.

In chapter 3 of this thesis, the anti-fibrotic potential of NK cells in the context of CHB was investigated. Data accumulating over the past decade point to a number of different roles for NK cells in CHB, in line with their capacity to exert both antiviral (Dunn et al., 2007; Vivier et al., 2011) and immunoregulatory functions (Martin-Fontecha et al., 2004; Morandi et al., 2006; Peppa et al., 2013; Rehermann, 2013). This study is the first to examine their role in CHB associated fibrosis.

2006 was a milestone year in the area of NK cell-HSC interactions. Two key independent studies reported for the first time the anti-fibrotic role of NK cells by demonstrating that they could kill activated HSC in animal models of CCl_4-induced fibrosis (Melhem et al., 2006; Radaeva et al., 2006). In 2012, this interaction was studied for the first time using primary human HSC and NK cells from HCV patients (Glassner et al., 2012).

This thesis has examined the NK cell-HSC interactions for the first time in the setting of CHB and has shown that *in vitro,* NK cells from CHB patients have the capacity to trigger apoptosis of HSC. However there is considerable variation in their potential to induce apoptosis and in the majority of cases the extent of apoptosis of HSC demonstrable *in vitr*o is limited, in line with the

progressive fibrosis seen in many of these patients. This varied potential of NK cells from CHB patients to kill HSC is possibly due to the altered phenotype of NK cells in CHB (Maini and Peppa, 2013; Rehermann, 2013). Also since this is a cross-sectional study, the time-point or the stage of the disease at which the patients are bled for this study may influence their capacity to induce apoptosis of HSC. Alternatively, these variations in the potential of NK cells of patients to trigger apoptosis of HSC could be a manifestation of inter-individual variation in host responses that may partially account for variation in disease progression. To examine this further the CHB patients enrolled in this study were stratified on the basis of HBV viral load, HBeAg status, degree of fibrosis and ALT levels. This division did not lead to a conclusive finding however, patients with high ALT or more fibrosis showed a trend towards a higher potential to kill HSC. This could suggest that during flares of liver inflammation characteristic of certain stages of chronic infection, NK cells become transiently activated and kill activated HSC. It was also observed that CHB patients taking oral anti-viral NUCs were unable to induce apoptosis of HSC, congruent with the observation that treatment with NUCs reduces the activation status of NK cells (Peppa et al., 2010), which in turn may have an impact on their anti-fibrotic activity.

To examine this further, NK cells were activated *in vitro* using recombinant IFN-α, a type-I IFN that has been previously described to activate NK cells (Bryceson et al., 2006; Caligiuri, 2008; Nguyen et al., 2002). This study revealed that IFN-α activated NK cells had an improved potential to trigger apoptosis of HSC; examining the changes in various NK cell markers

implicated upregulation of TRAIL in this augmentation. The fact that NK cells from patients with high ALT and those with augmented TRAIL expression had an improved potential to kill HSC is in line with previously published data that demonstrated that NK cells from CHB patients with high ALT have higher expression of TRAIL (Dunn et al., 2007; Peppa et al., 2010). The levels of TRAIL on NK cells have been shown to be further upregulated in CHB patients on peg-IFNα therapy (Micco et al., 2013). In HCV, it has been shown that patients treated with peg-IFNα therapy have a slower rate of progression of fibrosis (Vukobrat-Bijedic et al., 2014). The same effect has been observed in CHB (Papatheodoridis et al., 2005). However, it remains to be established if this is due to activation of NK cells. It has been demonstrated in *in vitro* experiments using LX2 that IFNα can directly negatively influence the proliferative potential of HSC (Ogawa et al., 2009). This thesis has focused of the effect of *in vitro* treatment of NK cells with IFN-α and their interaction with HSC. Several roles of the TRAIL pathway in NK cells from CHB patients have already been researched. It has been demonstrated that in CHB, NK cells can interact with infected hepatocytes via the TRAIL pathway to induce their apoptosis and play an anti-viral role while contributing to liver damage (Dunn et al., 2007). TRAIL-expressing NK cells also have the capacity to kill virus-specific CD8 T cells via the TRAIL pathway and thus may contribute to viral persistence (Peppa et al., 2013).

The finding that NK cells were able to kill stellate cells even in patients with increased levels of CHB-related liver fibrosis suggests that they are unable to prevent progressive fibrosis, even though they may still be constraining it to

some degree. It is also worth noting that during liver injury, activated HSC produce high levels of TGF-β compared to quiescent HSC (Jeong et al., 2011). TGF-β and other immunosuppressive cytokines such as IL-10 have been shown to dampen the effector function of NK cells; this could be another factor responsible for the varied potential of NK cell to kill in CHB (Dasgupta et al., 2005; Peppa et al., 2010; Yu et al., 2006). In murine studies, it has been demonstrated that TGF-β produced by HSC does influence anti-fibrotic activity of NK cells (Jeong et al., 2011). Another explanation could be that the variation in NK cell function is attributable to differential expression of inhibitory KIRs on NK cells of CHB patients that has not been studied in detail yet. The balance between activating and inhibitory KIRs on NK cells have been shown to influence their ability to kill HSC in HCV and murine fibrosis models (Muhanna et al., 2011).

Having established the role of the TRAIL pathway in NK cell mediated apoptosis of HSC, we further investigated NK cell - HSC interactions via the TRAIL pathway to further probe the role of this pathway in liver fibrosis. We demonstrated that even though *in vitro* augmentation of TRAIL on NK cells did improve their potential to kill HSC, complete apoptosis of HSC was never achieved even after the addition of high dose of *SuperKiller* TRAIL (positive control). Additionally, TRAIL expression on NK cells did not correlate with their ability to induce apoptosis of HSC and TRAIL blockade did not abrogate NK cell killing of HSC.

To understand this better, we further investigated the phenotype of HSC that may allow them to escape TRAIL and NK cell-mediated apoptosis. As has been explained in previous sections, TRAIL can interact with TRAIL-R1 and TRAIL-R2 to induce apoptosis and with TRAIL-R3 and TRAIL-R4 to suppress apoptosis. It has been previously described that both primary and cell line (LX2) HSC express high levels of TRAIL-R2 (Glassner et al., 2012; Taimr et al., 2003). However, the protein expression of TRAIL-R3 and -R4 on primary human HSC has been demonstrated for the first time in this thesis. Expression of TRAIL-R3 and TRAIL-R4 has been demonstrated in several tumor cells such as osteosarcoma cells, colon cancer cells, acute myeloid leukemia cells, breast cancer cells and other primary cells such as hepatocytes in HCV, CD8 T cells, colon epithelia, HUVECs at protein level directly *ex vivo* (Bouralexis et al., 2003; Brost et al., 2010; Brost et al., 2014; Clancy et al., 2005; Riccioni et al., 2005; Secchiero et al., 2003; Toscano et al., 2008). However to date, the studies that have investigated a functional role for TRAIL-R3 and TRAIL-R4 have done so using cell lines that have been manipulated to overexpress these receptors (Clancy et al., 2005; Davidovich et al., 2004; Merino et al., 2006; Sheridan et al., 1997; Walczak, 2013; Zhu et al., 2011). The 'decoy' role of these receptors has been demonstrated in several such overexpression studies showing that interaction of TRAIL with these receptors prevents apoptosis (Bouralexis et al., 2003; Davidovich et al., 2004; Falschlehner et al., 2007; Merino et al., 2006). Overexpression experiments have also revealed the potential role of TRAIL-R4 in promoting cell survival and proliferation (Degli-Esposti et al., 1997; Lalaoui et al., 2011). Studies have also demonstrated that TRAIL-R4 can interact with TRAIL-R2 in

a ligand dependent as well as independent manner to protect the cell from TRAIL-mediated apoptosis (Clancy et al., 2005; Merino et al., 2006). To our knowledge, our work constitutes the first report of the functional apoptosis-inhibitory role of TRAIL-R3 and TRAIL-R4 on primary human cells. This study has demonstrated that blocking these receptors on HSC makes them more susceptible to NK cell-mediated apoptosis, further underscoring the crucial role of these receptors in protecting activated HSC from undergoing apoptosis. We have thereby demonstrated that this 'decoy' effect has a physiological role in primary human cells against NK cells analysed directly ex vivo from patients with CHB.

We have shown that the expression level of TRAIL-R4 on HSC has a clear negative correlation with their potential to undergo TRAIL mediated apoptosis. Blocking TRAIL-R4 on HSC and co-culturing them with NK cells activated with IFN-α further increased their susceptibility to undergo apoptosis. This was also observed while blocking TRAIL-R3 in some of the cases.

A possible explanation for the effect of TRAIL-R4 blockade is that interaction between TRAIL and TRAIL-R4 could result in signaling via the truncated trans-membrane domain of TRAIL-R4, promoting cell-survival and proliferation via the NFκB and PI3/Akt pathway (Degli-Esposti et al., 1997; Lalaoui et al., 2011). Blocking this regulatory receptor not only allows TRAIL to preferentially bind to other receptors but also inhibits the survival pathway; as a result when TRAIL engages with TRAIL-R2 there is no competitive/regulatory mechanism to check the initiation of the caspase

cascade. However, when TRAIL-R3, is blocked, (which completely lacks the intracellular death domain and is known to work by competition (Merino et al., 2006)), TRAIL can still engage with apoptosis-inducing TRAIL-R2 and pro-survival TRAIL-R4, which might therefore be less effective at enhancing NK mediated killing. These downstream pathways have not been studied in HSC, however these are plausible mechanisms by which TRAIL-R4 may protect HSC from TRAIL-mediated apoptosis and promote HSC proliferation and survival has illustrated in Figure 6.1.

Results from this thesis suggest that NK cells from CHB patients could play a regulatory role in liver fibrosis (Figure 6.2). Depending on the interaction of TRAIL expressed on NK cell with the TRAIL receptors expressed on HSC, there could be three plausible fates of HSC. The TRAIL-TRAIL-R2 interaction could lead to apoptosis of HSC, therefore having an anti-fibrotic effect. TRAIL on NK cells could also interact with TRAIL-R3 and -R4 and prevent apoptosis of HSC and thus have a pro-fibrotic effect. The third possibility that remains to be explored further is that TRAIL on NK cells could interact with TRAIL-R4 on HSC and promote the proliferation and survival of HSC via Akt and NFκB signaling, again having a pro-fibrotic role.

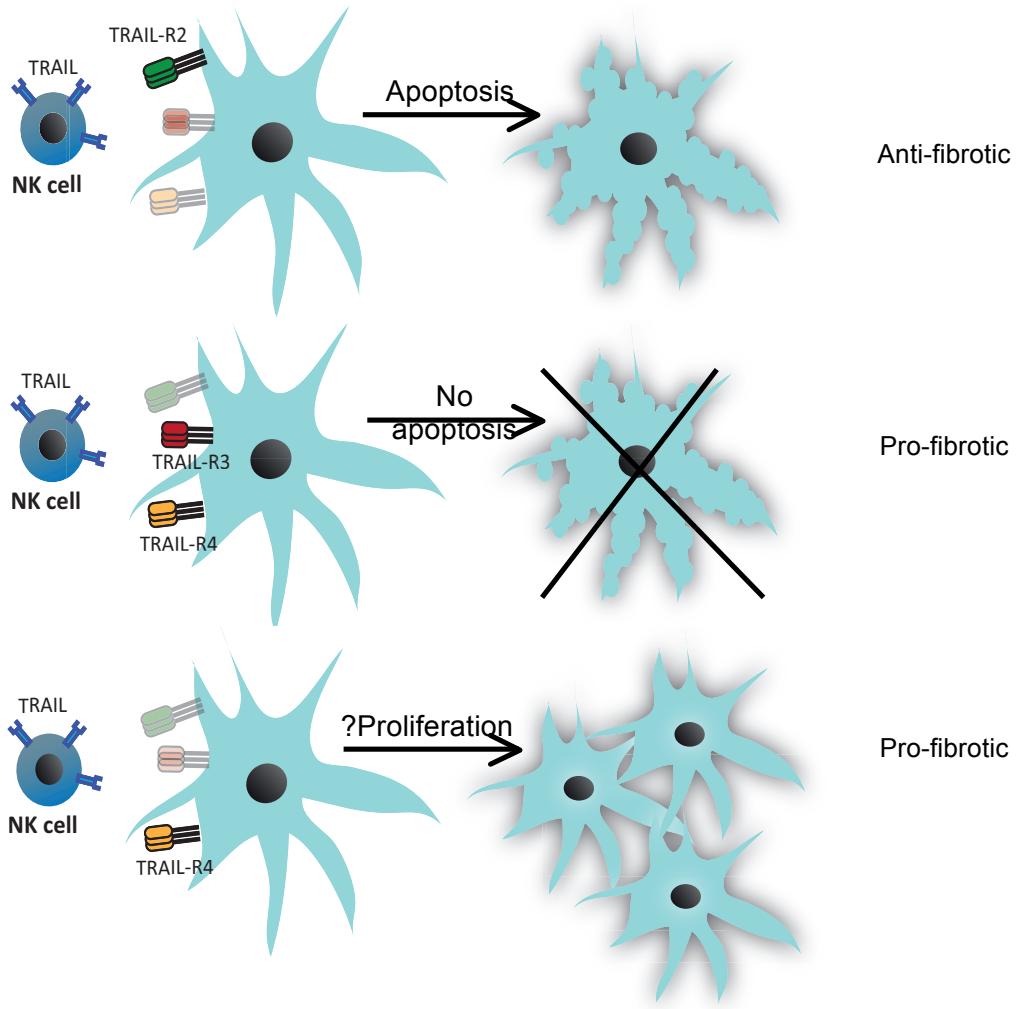


Figure 6-1: Potential outcomes of NK cell-HSC interaction by TRAIL- TRAIL receptor pathway.

Diagram representing 3 putative effects of TRAIL engagement with TRAIL-receptors on HSC. If TRAIL on NK cell interacts with TRAIL-R2 on HSC, it can promote apoptosis of HSC and play an anti-fibrotic role (top panel). TRAIL could interact with TRAIL-R3 and -R4 to allow HSC to escape apoptosis and therefore have a pro-fibrotic role (middle panel). TRAIL on NK cells might also interact with TRAIL-R4 on HSC to promote HSC proliferation and in turn have a pro-fibrotic role (bottom panel).

The balance between these different outcomes, dictated by NK TRAIL expression and primary HSC TRAIL receptor expression, may be an important determinant of liver fibrosis progression.

The unanswered questions arising from this thesis and the future and translational potential of this thesis are discussed further in the next section.

Questions and future directions

This thesis has contributed to the field of study of the regulatory role of NK cells in CHB as well as the area of the apoptosis-resistant nature of HSC that promotes fibrosis. The findings of the two results chapters have generated some interesting questions which merit further study. These are:

- Which mononuclear cell is responsible for activating HSC and does this have a pro-fibrogenic effect?
- Are PBMC from CHB patients able to activate primary human HSC?
- Is the anti-fibrotic effect of peg-IFNα therapy in CHB mediated through NK-HSC interactions?
- Besides TRAIL, are there other NK-HSC cells interactions that might promote/inhibit fibrosis?
- Does TRAIL-TRAIL-R4 interaction influence the proliferation of HSC?

Although this is not an exhaustive list, answering these questions will help to enhance the understanding of these cells and the mechanisms through which they might be implicated in liver fibrosis. This will allow us to understand and identify pathways and molecules that might represent therapeutic targets for the treatment of CHB associated liver fibrosis. The tenable answers to these

questions have been discussed with some preliminary data (where available) below.

Which mononuclear cell is responsible for activating HSC and is this a pro-fibrogenic effect translated on to primary human HSC?

In chapter two of this thesis, it was demonstrated that PBMC from CHB patients have a capacity to activate HSC (LX2) at mRNA and protein levels by secreting soluble mediators. Next, it would be interesting to identify which cell(s) is responsible for the production of these soluble mediators. This can be done by using magnetic bead purification or antibody labeled cellular sorting to isolate individual cells that comprise the PBMC population such as CD4+ T cells, CD8+ T cells, NK cells, T regs, B cells, NKT cells, macrophages and dendritic cells and culture them with HSC in a transwell system and then study the activation of HSC, post transwell based co-culture. It is likely that more than one cell type is playing a role in activating HSC. The interaction of different cell types with each other could also be contributing to their production of HSC-activating molecules. Once the cell types are identified it would be easier to narrow down and identify the secreted compound by the cell that is playing an activatory role towards HSC.

Are PBMC from CHB patients able to activate primary human HSC?

These findings from the study in this thesis have been shown in LX2, an immortalized HSC cell line. While LX2 form a good constant in the experimental setup which has PMBC from different participants as the variable, in the future it would be interesting to apply this experimental set up to primary human HSC to determine whether these findings are also observed in a more physiological setting.

Is the anti-fibrotic effect of peg-IFNα therapy in CHB mediated through NK-HSC interactions?

In vitro activation of NK cells can increase their potential to kill HSC. It would be interesting to investigate the capacity of NK cells isolated from patients on peg-IFNα therapy to induce apoptosis. It has been previously published that NK cells of patients on peg-IFNα therapy have increased levels of TRAIL expression on their surface along with improved effector function (Micco et al., 2013). This has not been investigated in this study but would be the next step and would be interesting to explore in the future. It has been reported that HCV patients on peg-IFNα therapy have a slower rate of progression of fibrosis and NK cells have been shown to play a role in this (Glassner et al., 2012; Vukobrat-Bijedic et al., 2014). In CHB as well, it has been demonstrated that patients who respond to peg-IFNα therapy have a slower rate of progression of fibrosis (Papatheodoridis et al., 2005). The role of NK cells of these CHB patients on peg-IFNα therapy in the context of reduced rate of progression of fibrosis would be the next step of investigation. For this, PBMC from CHB patients would be isolated at time-points prior to treatment and during treatment with peg-IFNα therapy. NK cells isolated from these PBMCs would then be co-cultured with HSC to determine changes in their capacity to induce apoptosis. At the time points at which the patients are bled for the study, their fibrosis status could also be checked using a non-invasive biomarker test (ELF test) to correlate the fibrosis stage in the patient with the potential of their NK cells to mediate apoptosis.

Besides TRAIL, could NK cells-HSC interact via other pathways that might promote/regress fibrosis?

In the past decade, several murine studies have investigated the various mechanisms through which NK cells can kill HSC. It has been demonstrated that both murine and human HSC express some of the ligands for NKG2D, including ULBP-2 and MICA/B. NKG2D is highly expressed on NK cells of healthy individuals, and this high expression is maintained on NK cells of CHB patients (Maini and Peppa, 2013). In HCV studies and animal studies it has been shown that this pathway contributes to NK cell mediated killing of HSC (Glassner et al., 2012; Radaeva et al., 2006). Along with the NKG2D pathway, the role of the Fas-FasL pathway has also been elucidated. HSC express Fas and can interact with FasL on NK cells to undergo apoptosis (Glassner et al., 2012). Along with these pathways, recently the role of NKp46 in killing HSC has been described. It has been discovered using fusion proteins that HSC express on their surface the unknown ligand for NKp46, and can interact via this pathway to kill HSC (Gur et al., 2012). Expression of NKp46 on NK cells of CHB is higher compared to healthy controls and this is further augmented in CHB patients on peg-IFNα therapy (Micco et al., 2013; Rehermann, 2013). Whether any of these pathways contributes to killing of HSC in CHB still remains to be investigated. These various pathways are illustrated in the diagram below (Figure 6.4).

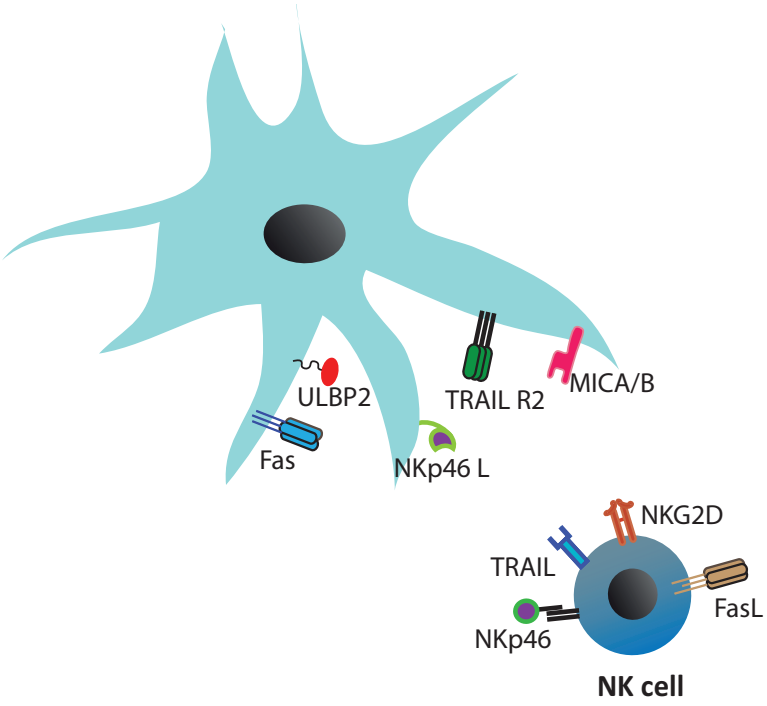


Figure 6-2: NK cell-HSC death inducing interactions.
Diagramatic representation of all the known pathways by which NK cells can kill HSC: Fas-FasL, NKG2D-MICA-B/ULBP-2, NKp46-NKp46L and TRAIL-TRAIL R2 (Gur et al., 2012; Radaeva et al., 2006).

Killer immunoglobulin-related receptor (KIR) are expressed on NK cells. NK cells express both activatory KIRs (aKIRs) and inhibitory KIRs (iKIRs). Both types of KIRs on NK cells can interact with MHC Class I molecules on the target cell to either promote or inhibit their killing (Locatelli et al., 2013). Murine studies have shown that the aKIR:iKIR expression ratio is crucial in determining the HSC killing potential of NK cell (Melhem et al., 2006). Subsequent murine studies have also demonstrated that silencing iKIRs on NK cells improves their potential to kill HSC (Muhanna et al., 2011). The expression levels of aKIRs and iKIRs on NK cells of CHB patients remain to

investigated. This could be another possible pathway that influences the killing potential of NK cells against HSC.

In animal studies HSC have been shown to downregulate their MHC Class I (Melhem et al., 2006). However we observed expression of HLA-E on HSC as illustrated in Figure 6.5. HLA-E interacts with CD94/NKG2 complex including inhibitory receptor NKG2A on NK cells (Cheent et al., 2013; Sullivan et al., 2007). NKG2A has been shown to be upregulated on NK cells of CHB patients on peg-IFNα therapy (Micco et al., 2013). This could plausibly be a mechanism by which NK cells – HSC cross talk may down-modulate NK cell killing by re-programming the balance of activatory and inhibitory signals.

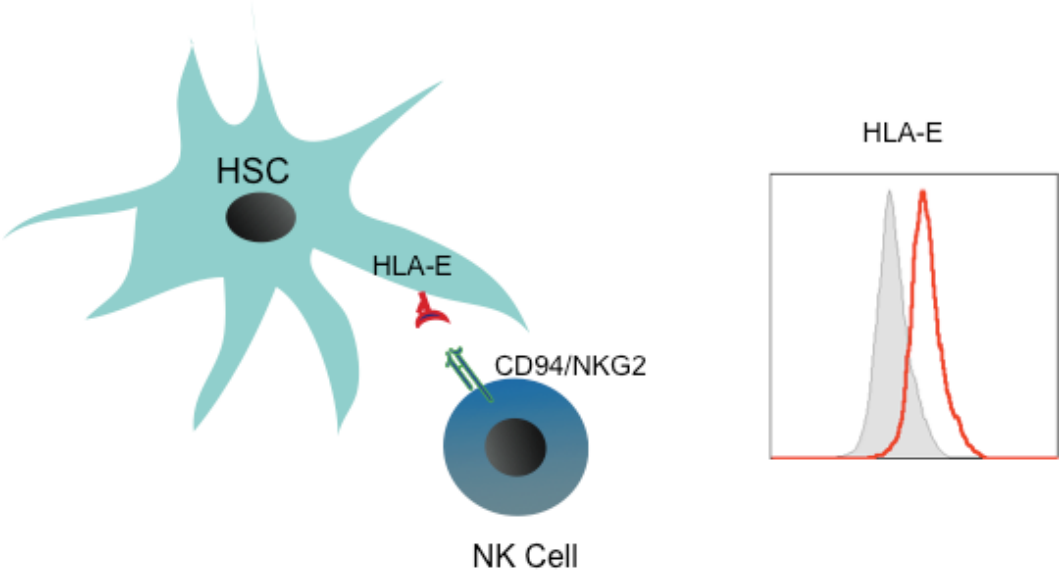


Figure 6-3: Primary human HSC express HLA-E.
HLA-E on HSC could interact with inhibitory CD94/NKG2 complex on NK cells to modulate NK cell effector function. FACS histogram showing the expression of HLA-E on primary human HSC in red against matched isotype control in grey.

Reflecting on the results from this thesis, it can be stated that regardless of which of these pathways contribute to the killing of HSC in CHB, none of them are able to completely prevent progression of fibrosis. HSC hold the potential to escape apoptosis and contribute to fibrosis.

Does the TRAIL - TRAIL-R4 interaction influence the proliferation of HSC?

To investigate if the interaction of TRAIL with TRAIL-R4 on HSC influenced the proliferation of HSC, HSC were transduced with lentivirus containing short hairpin (sh) against TRAIL-R4 to knock-down its expression and then examine the proliferation potential of these cells.

Using this system, we would like to examine the role of TRAIL-R4 in proliferation and survival of HSC. For this we will transduce cells, knock-down the expression of TRAIL-R4 and then compare their proliferative potential using the colorimetric MTT assay. We would investigate whether this is ligand dependent or independent by knocking down TRAIL-R2 (to avoid TRAIL-mediated apoptosis) on HSC and then adding TRAIL in a dose dependent manner to the system and determining the proliferation of these cells using the Ki67 proliferation assay and analysing data using flow cytometry.

Future Outlook:

The causes and factors initiating liver fibrosis in HBV infection and other liver etiologies have been researched over the past couple of decades. The conditions that perpetuate and sustain the pace of progression of fibrosis still remain to be fully explained and are an area of active research. This thesis has been able to identify some of the mechanisms that aid progression of fibrosis: the inability of NK cells from CHB patients to efficiently kill HSC and anti-apoptotic features of HSC. These pathways still need to be investigated further. They represent potential targets for novel therapies. It will be particularly important to study the capacity of activated NK cells from CHB patients on peg-IFNα to kill HSC.

Along with activating patient NK cells, studying the characteristics of an individual's HSC may be able to help predict their chances of developing liver fibrosis and the rate at which it will progress. For this, histological expression of apoptosis inducing TRAIL-R2 and apoptosis inhibiting TRAIL-R3 and TRAIL-R4 on HSC of patients with CHB could be studied on liver sections from diagnostic biopsies, allowing direct ex vivo assessment of a larger cohort. This would allow us to examine whether the expression of these receptors correlated with the degree of fibrosis in these patients. If a correlation was found, in the future a longitudinal study could be setup to study the histological expression of these receptors on patient HSC together with the capacity of their NK cells to mediate apoptosis of HSC to see if these predicted the rate of fibrosis progression over the following year or more of

follow-up. If this longitudinal study gave promising results, expression of TRAIl-R2, TRAIL-R3 and TRAIL-R4 on HSC and the phenotype of NK cells of an individual could potentially be used as future biomarkers of fibrosis progression. It is noteworthy that this approach could potentially be of utility for any liver disease leading to fibrosis and not just CHB.

Another ambitious yet possible approach to understand stellate cell biology better could be to target HSC to downregulate their anti-apoptotic receptors. This could potentially be achieved by transducing HSC in an *in vivo* model with lentivirus containing short hairpin to knock-down TRAIL-R4 and/or cFLIP. We have demonstrated that HSC can be successfully and effectively transduced with lentivirus. An adjuvant could be attached to the lentivirus that targets them specifically to HSC. In this way activated HSC could be targeted to knock-down TRAIL-R4 and cFLIP expression in order to promote their sensitization to apoptosis by NK cells, and possibly also to reduce their proliferative potential.

However therapeutic manipulation of stellate cells would need to take into consideration their other putative functions. Other than their indispensible role in fibrogenesis, HSC play other homeostatic roles in maintaining an effective framework to facilitate liver tissue repair in case of acute injury. In a quiescent state they also form the reservoir of vitamin A storage in the body. HSC also play a role in regulating the liver vasculature; activation of HSC has been shown to interfere with the normal sinusoidal endothelial fenestrations by depositing extracellular matrix and create an imbalance between

vasoconstriction and dilation that leads to increased hepatic vascular resistance, further leading to intrahepatic portal hypertension (Iwakiri et al., 2014). HSC may be able to present antigen to T cells (Winau et al., 2007). HSC also play a key immunomuodulatory role since they have been demonstrated to regulate the expansion of immune cells such as myeloid derived suppressor cells (MDSC) and T regs, which can exert crucial immunoregulatory functions (Chou et al., 2011; Dunham et al., 2013; Hochst et al., 2013).

From the work presented here, the TRAIL pathway emerges as one mechanism by which the fate of hepatic stellate cells is tightly regulated. An exciting area of future work will be to further define the importance of this alongside other interactions between stellate cells and liver-resident NK cells and the potential to apply this as a biomarker or therapeutic target in liver fibrosis.

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